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(57) Abstract

A method of diagnosing bladder cancer in a human patient comprising the steps of (i) obtaining a sample containing nucleic acid and/or protein from the bladder of a patient, preferably from the urothelium; and (ii) determining whether the sample contains a level of Pax 5 nucleic acid or protein associated with bladder cancer. A method of treating bladder cancer comprising the step of administering to the patient an agent which selectively prevents the function of Pax 5. A genetic construct comprising a nucleic acid encoding a molecule capable of preventing the function of Pax 5 expressed in a urothelial cell. The methods and compositions are particularly suited to transitional cell carcinoma of the bladder.

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DIAGNOSIS AND TREATMENT OF CANCER

The present invention relates to methods of determining whether a patient has bladder cancer and how advanced or invasive that cancer is; and it relates to methods of treating bladder cancer.

Cancer is a serious disease and a major killer. Although there have been advances in the diagnosis and treatment of certain cancers in recent years, there is still a need for improvements in diagnosis and treatment.

Cancer is a genetic disease and in most cases involves mutations in one or more genes. There are believed to be around 200,000 genes in the human genome but only a handful of these genes have been shown to be involved in cancer. Although it is surmised that many more genes than have been presently identified will be found to be involved in cancer, progress in this area has remained slow despite the availability of molecular analytical techniques. This may be due to the varied structure and function of genes which have been identified to date which suggests that cancer genes can take many forms and have many different functions.

About 90% of bladder cancer is transitional cell (urothelial) carcinoma, with the remainder being sqamous cell carcinoma (5 to 7%), adenocarcinoma (about 2%), small cell and spindle cell carcinoma (both very rare). Carcinomas may be of mixed cell type.

Transitional cell carcinoma may be further classified depending on the growth pattern into the following types: papillary (about 70%), papillary and infiltrating (about 20%), sessile, nodular and infiltrating (about 10%) and non-papillary, non-invasive ie carcinoma *in situ* (CIS; about 1 to 5%).

Transitional cell carcinoma is rare under the age of 40 or 50; most patients are between the ages of 50 and 70, with the peak death rate between 65 and 75 years. Males have a two- to fivefold greater incidence than females. Transitional cell carcinoma is very rare under the age of 20.

Bladder cancer appears to result mainly from exposure to environmental carcinogens. Aromatic amines, including benzidine, 2-napthylamine and para-aminodiphenyl are thought to be involved. The development of cancer results from long exposure (2 to 20 years) and has a latent period of 5 to 30 years. High risk occupations include the dye (aniline), rubber and leather industries. Smoking may also increase the risk: the incidence of bladder cancer is two- to threefold higher in smokers. Dietary factors such as tryptophan and artificial sweeteners may be involved, and bracken fern in animals, for example cattle. Phenacetin, cyclophosphamide and radiation may also promote bladder cancer.

Urinary tract diseases, for example bladder lithiasis, chronic infection, diverticula, schistosomal infection (linked with sqamous cell carcinoma) malformations (associated with adenocarcinomas) may also be associated with an increased risk of bladder cancer. Catheterisation damage has not been linked with predisposition to bladder cancer, but long term bladder stones may predispose to sqamous cell bladder cancer.

The major source of invasive and metastasising carcinomas in humans is thought to be non-papillary tumours, ie CIS. p53 expression, which may be linked with malignancy, may be more common in CIS than other transitional cell carcinomas. CIS is thought to develop from urothelial dysplasia. Invasive and metastasising carcinomas may arise from other

forms of transitional cell carcinomas, but a greater proportion of CIS than other forms of TCC may lead to invasive and metastasising carcinomas. In experimental animals, non-invasive papillary tumours appear to arise most frequently from exposure to carcinogens. Experimentally induced invasive carcinomas appear to arise from squamous metaplasia.

Several areas of molecular research have contributed to the understanding of the initiation and progression of transitional cell carcinoma of the bladder (urinary bladder). Approximately 50% of bladder tumours exhibit loss of p53 function and this is strongly associated with higher tumour stage and grade (14).

Similarly, retinoblastoma gene mutations are seen in about 30% of bladder cancer with propensity to more advanced disease (15, 16). DNA ploidy has also offered some predictive value in superficial disease (17). It has long been thought that deletions on chromosome 9 are an initiating event in urothelial transformation. Comparisons of loss of heterozygosity in tumour and lymphocyte DNA from the same patient has shown that 67% of all bladder tumours, in all stage and grades, have deletions on chromosome 9 (10). The putative tumour suppresser gene lost in these deletions is located at 9p21 and this region is commonly deleted in a variety of tumours (11).

Many markers of initiation and progression have been investigated, but as yet, none can predict outcome as well as the pathologist. Management of bladder cancer is reviewed, for example, in Soloway (1992) "Managing superficial bladder cancer: an overview" *Urology* **40(6 suppl)**, 11-22.

Both normal and malignant urothelium shed cells into the urine and these cells are routinely collected for cytological analysis in the aim of diagnosing malignancy non-invasively. Invasive investigations include procedures such as flexible cystoscopy. A test which may be carried out on urine is the Bard BTA stat test, described, for example, in Sarosdy *et al* (1997) *Urology* **50(3)**, 349-353. This test detected 67% of recurrent cancers in the above study and was held to be superior to cytological analysis of cells shed into the urine.

Transurethral resection biopsies may also be carried out and may be classified by a trained histopathologist using standard TNM and grade classifications, as discussed in Sobin & Flemming "TNM classification of malignant tumours, fifth edition (1997) Union International Contre le Cancer and the American Joint Committee on Cancer" *Cancer* **80(9)**, 1803-1804 and Chen *et al* (1996) "The significance of tumour grade in predicting disease progression in stage Ta transitional cell carcinoma of the urinary bladder" *Br J Urology* **78(2)**, 209-212. For example, stage pTa indicates that the tumour is confined to the mucosa, pT1 indicates that the tumour has invaded the lamina propria, pT2, pT3a and pT3b indicate that the tumour has invaded superficial muscle, deep muscle and perivesical fat respectively. pT4a indicates metastasis to adjacent organs, pT4b indicates fixation to pelvic or abdominal wall, N^+ indicates involvement of regional lymph nodes, while M^+ indicates metastatic lesions other than lymph nodes. Thus the invasiveness/metastasis of the tumour may be indicated by its stage. An alternative classification is the Jewett-Strong-Marshall scheme, which recognises similar stages to those listed above.

Regardless of cell type, clinicopathologic stage is the most important parameter in planning treatment and prognosis. Clinical staging may be inaccurate when compared with pathologic staging due to the inability to assess depth of invasion and regional lymph node involvement.

The main clinical sign of bladder cancer is macroscopic hematuria. Other symptoms include irritative bladder symptoms similar to cystitis (for example frequency, dysuria, urgency and suprapubic discomfort) but with no sign of infection. Local invasion may give rise to other symptoms arising from the compression of nerves, ureters and veins. For CIS, hematuria is present in only about 20% of patients. A history of transurethral resection of the prostate for these symptoms is common. The intensity of symptoms usually reflects the extent of the mucosal disease. Disease without symptoms is found by cytologic diagnosis in no more than 10% of patients.

CIS may be a multifocal or panurothelial lesion. The base of the bladder and trigone, including the ureteral orifices, are the most common locations. Ureteral CIS is found at cystectomy in 6% to 60% of cases and is most common in the juxtavesical segments, but has a low rate of progression (about 3%). There is, however, an increased risk of upper urinary tract tumours with multifocal CIS and distal ureteral involvement: new tumours develop at an average of 61 months. Involvement of prostatic ducts and the urethra, which occurs in 18-45% of cases, may cause pseudoprostatitis and penile voiding symptoms, respectively.

CIS is the initial presenting form of urothelial malignancy in approximately 1% of cases: unexpected microinvasion may be found in up to one-third of cystectomy specimens. Recurrences of CIS are common

following transurethral resection of a localised lesion: 30% occur in the first three months, 60% by six months and 85% by one year. About 50% of patients with primary CIS develop invasive carcinoma in four to six years, and 80% do so within ten years. More commonly, CIS accompanies synchronous or metachronous papillary tumours or frankly invasive non-papillary transitional cell carcinoma.

Approximately 70% of all bladder cancers are localised to the bladder at the time of diagnosis, and less than 10% show clinical evidence of distant metastases. Five-year survival rates for patients with invasive bladder cancer following cystectomy plus adjuvant radiation therapy are 35%-52%; distant metastases occur in 30-50% of patients, usually within 12-18 months. The most common metastatic sites, in addition to regional lymph nodes, are lungs, liver and bone.

Transitional cell carcinoma of the bladder forms a very heterogeneous group of neoplasms. The prognosis is greatly worsened by muscle-invasive disease at presentation (stage pT2 and above, according to the TNM classification; Sobin & Fleming, cited above) and over half of those with superficial disease (stages pTa and pT1) will develop recurrent tumours (Soloway (1992) *Urol* **26**, 30-31), usually of the same stage and grade. Furthermore, ~20% of these superficial tumours will progress to muscle invasion; ~4% of Ta and 30% of T1 tumours will progress, according to the National Bladder Cancer Group (Koontz (1985) *Urol* **26**, 30-31). Chen *et al* (1996) *Br J Urol* **78**, 209-212 showed that in Ta tumours, progression was more prevalent in grade 3, poorly differentiated tumours (28.5%) than in grade 2 (9.0%) and grade 1, well-differentiated tumours (2.1%).

Intravenous pyelography is capable of revealing tumours as small as 4-5 mm. Characteristics of the filling defect that are important in making the diagnosis include fixation ie implantation of the bladder wall, and irregular outlines. Intravenous pyelography is also useful in demonstrating lack of bladder distensibility, hydroureteronephrosis, or a non-functioning kidney, as well as in evaluating the upper urinary tract for urothelial tumours. In patients who have had no prior bladder surgery, radiation therapy or recent transurethral resection, the computed tomography (CT) scan may be helpful.

Present screening methods are therefore unsatisfactory; there is no reliable method for diagnosing the cancer, predicting the outcome or preventing invasiveness and possible metastatic spread.

Pax genes are a family of nine developmental control genes coding for nuclear transcription factors. They play an important role in embryogenesis and are expressed in a very ordered temporal and spatial pattern. They all contain a "paired box" region of 384 base pairs encoding a DNA binding domain of about 128 amino acids which is highly conserved throughout evolution (8). The influence of Pax genes on developmental processes has been demonstrated by the numerous natural mouse and human syndromes that can be attributed directly to even a heterozygous insufficiency in a Pax gene (8). Their role in controlling cell growth and proliferation in such a regulated fashion has lead to the investigation of their involvement in the deregulated growth seen in neoplasia.

The subgroup of Pax 2, 5, and 8 (class III) are expressed latest in development and only in the undifferentiated, highly mitotic cells of the

ventricular zone of the CNS (Pax 2, 5 and 8), the developing kidney (2 and 8), B-cell progenitors (5) and the thyroid (8). In mice, after birth, Pax 5 is expressed exclusively in B cells and testis (9).

Because of the powerful effect of these transcription factors on cellular growth and differentiation and the salient feature of expression for Pax 2, 5 and 8 in highly mitotic, undifferentiated cells they are candidates for proto-oncogenes. Pax genes are capable of transforming fibroblasts and producing solid, vascular tumours in nude mice (1). They have been shown to be inappropriately expressed in many different tumours eg glioblastoma (Pax 5), renal cell carcinoma (Pax 2), medulloblastoma, non-Hodgkins lymphoma (Pax 5), Wilm's tumour (Pax 2 and 8) and rhabdomyosarcoma (Pax 3 and 7) (reviewed in (7)). Certainly, *in vitro* studies have shown that antisense to Pax 2 in renal cell lines expressing this gene considerably hinders growth. Pax 2 has an important role in the pathogenesis of Wilm's tumour and renal cell carcinoma and is transcriptionally repressed by the Wilm's tumour suppressor gene. EP 0 655 926 relates to the involvement of Pax genes in cancer.

As reviewed in (8), Pax 5 is inappropriately expressed in highly malignant astrocytomas, but the mechanistic basis for Pax 5 functioning as a proto-oncogene in these tumours is not known. Pax 5 was expressed only in discrete cell populations within the tumour. These areas also expressed various other oncogenes -Myc, Fos, Jun - and the epidermal growth factor receptor, suggesting the possibility of Pax genes co-operating in a pathway that involves other known oncogenes and tumour suppressor genes.

Pax 5 may regulate the B-cell specific CD19 gene. A homologue in sea urchin, TSAP, may regulate the histone H2A-2 and H2B-2 genes. The

ability of Pax genes to induce transformation and tumours suggests that possible target genes are involved in coding for proteins that are involved in the regulation of the cell-cycle or components of signal transduction mechanisms. Possible target genes could be induced or suppressed. Pax gene expression has been identified in undifferentiated cells and thus potentially induced genes may contribute to the undifferentiated state, whereas suppressed genes may contribute to the differentiated state.

It is suggested by Stuart *et al* (23) that Pax 5 may mediate transcriptional repression of the p53 tumour repressor. There appears to be a conserved Pax 5 binding site within the untranslated first exon of the p53 gene. Mutation of this region appears to render the p53 promoter inactive in cells and stops Pax 5 binding *in vitro*. It is postulated that the binding of Pax 5 to this site may impede transactivation of the promoter by an as yet unidentified positive regulatory factor.

Despite considerable research, a genetic marker that is useful in reliably predicting the outcome of bladder cancer has not been identified. It is now shown that Pax 5 expression has a role in the pathogenesis of bladder cancer, particularly transitional cell carcinoma of the bladder. Three established transitional carcinoma cell lines and twenty-nine primary transitional cell carcinomas were investigated using reverse transcription polymerase chain reaction to detect Pax 5 expression. Pax 5 is expressed in a higher proportion of tumours the higher the stage and grade of the disease. Pax 5 may be an important diagnostic marker of bladder cancer.

Pax 5 expression may also be useful in assessing non-malignant pathological processes, such as urothelial damage, for example cell

proliferation of the urothelium in response to an indwelling catheter. Such cell proliferation may have a macroscopic resemblance to bladder tumour.

As noted above, Pax 5 is a known protein (although not previously known to be associated with bladder cancer) and the amino acid sequence of the protein, and nucleotide sequence of the cDNA of the mRNA encoding it are known (13). The mouse gene was isolated by Walther *et al* (1991) *Genomics* **11**, 424-434.

It is an object of the invention to provide methods useful in providing diagnoses and prognoses of bladder cancer, especially transitional cell carcinoma of the bladder, and for aiding the clinician in the management of bladder cancer. In particular, an object of the invention is to provide a method of assessing the invasive potential of bladder cancer, particularly transitional cell carcinoma of the bladder.

Further objects of the invention include the provision of methods of treatment of bladder cancer, in particular transitional cell carcinoma of the bladder, for example using inhibitors of Pax 5 expression or activity, for example using anti-sense based therapy.

A first aspect of the invention provides a method of determining the susceptibility of a patient to bladder cancer comprising the steps of (i) obtaining a sample containing nucleic acid and/or protein from the urothelial cells of a patient; and (ii) determining whether the sample contains a level of Pax 5 nucleic acid or protein associated with bladder cancer.

It will be appreciated that Pax 5 may be a pre-malignant marker, such that a change in the expression of Pax 5 may precede other signs of malignancy. Thus if an elevated level of Pax 5 is found in a sample from a patient that has no visible or easily detected signs of bladder cancer, this may indicate that the patient is in the early stages of developing bladder cancer or that the patient may later develop bladder cancer or be particularly susceptible to risk factors for developing bladder cancer, as listed above, for example smoking.

A second aspect of the invention provides a method of diagnosing bladder cancer in a patient comprising the steps of (i) obtaining a sample containing nucleic acid and/or protein from the urothelial cells of the patient; and (ii) determining whether the sample contains a level of Pax 5 nucleic acid or protein associated with bladder cancer.

It will be appreciated that determining whether the sample contains a level of Pax 5 acid or protein associated with bladder cancer may in itself be diagnostic of bladder cancer or it may be used by the clinician as an aid in reaching a diagnosis.

For example, in relation to bladder cancer, it is useful if the clinician considers the presence of blood in the urine, cytology of urinary voided cells, cytoscopy and biopsy as well as considering the level of Pax 5 in making a diagnosis. Histological analysis of biopsy may be useful in distinguishing non-malignant urothelial damage from malignancy. Radiologic investigation, for example intravenous pyelography may also be useful.

A third aspect of the invention provides a method of predicting the relative prospects of a particular outcome of a bladder cancer in a patient comprising the steps of (i) obtaining a sample containing nucleic acid and/or protein from the urothelial cells of the patient; and (ii) determining whether the sample contains a level of Pax 5 nucleic acid or protein associated with bladder cancer. Thus, the method may be useful in prognosis or aiding prognosis. Similarly to above, cytology, cytoscopy, biopsy and pyelography may be useful in making a prognosis of disease outcome.

It is preferred that the patient is a human patient. It is preferred that the sample is obtained using non-invasive procedures. It is particularly preferred that the sample is urine, into which urothelial cells (which may be malignant or non-malignant) may have been shed, for example naturally, or urothelial cells recovered from the patient's urine. It is preferred that the bladder cancer is transitional cell carcinoma of the bladder.

It will be appreciated that a sample, for example a biopsy sample, may contain more than one cell type, for example it may contain urothelial cells, blood, muscle, fibrocytes, supporting cells and squamous cells. Squamous cells and columnar cells, for example, may be shed into the urine along with urothelial cells. Blood cells and other cell types may also be present in the urine, for example as a consequence of bladder cancer. Malignant cells may be shed into the urine in greater numbers than non-malignant cells. Thus, higher grade tumours may shed more cells than lower grade tumours or normal bladder.

It will be appreciated that squamous cells, for example, may give rise to bladder cancer. It will be further appreciated that the methods of the invention may apply to squamous cells and squamous cell carcinoma of the bladder and to other cancers of the bladder. It is preferred however that the cancer is transitional cell carcinoma of the bladder.

It will be appreciated that quantification of Pax 5 expression may not be informative, for example in some samples containing more than one cell type. It may be sufficient to determine whether Pax 5 expression may be detected in a sample or not, for example using the methods (for example, RT-PCT based methods with detection by ethidium bromide staining) described in example 1, without further quantification. In normal tissue, Pax 5 expression may not be detected, whereas in malignant tissue Pax 5 expression may be detected, using detection methods analagous to those by which expression of the ubiquitous enzyme GAPDH (glyceraldehyde phosphate dehydrogenase) may be detected, for example using the methods described in example 1. Thus, in the methods of the invention a level of Pax 5 nucleic acid or protein associated with bladder cancer may be a level of Pax 5 nucleic acid or protein that may be detected by a method by which Pax 5 nucleic acid or protein is not detected in normal tissue, for example as described in example 1. It will be appreciated that a sample known to contain Pax 5 nucleic acid or protein, for example a previously tested malignant bladder cancer biopsy sample or a sample of a cell line or B-lymphoblastoid cells, for example as tested in example 1, may be used as a reference sample. A second reference sample may be a previously tested normal bladder biopsy sample or sample of a cell line in which Pax 5 has previously not been detectable. U937 cells may be suitable as such a reference negative sample.

It will be appreciated that the methods of the invention may be performed upon one or more individual cells. Thus, the level (which may be the detectable presence/absence) of Pax 5 expression may be assessed in individual cells. The results of the method may be expressed, for example, as a presence of Pax 5 in any of the cells tested, absence of Pax 5 in all of the cells tested, presence of Pax 5 in all of the cells tested, proportion of the cells tested in which the presence of Pax 5 was detected, or a numerical average of the quantified level of Pax 5 across all the cells tested, or across those cells in which Pax 5 expression was detected.

It will be appreciated that the sample in which the presence and/or level of Pax 5 is detected may be a portion of a sample obtained from a patient. For example, the sample in which the presence and/or level of Pax 5 is detected may be one or more cells obtained from a biopsy or urine sample obtained from a patient.

It will be appreciated that determination of the level (which includes determination of presence, in an amount sufficient to be detected, or absence) of the said Pax 5 in the sample will be useful to the clinician in determining how to manage the cancer in the patient. For example, since elevated levels (for example levels which may be detected, as opposed to levels in normal tissue may be too low to be detected) of the said Pax 5 may be associated with invasiveness or invasive potential, particularly in a transitional cell carcinoma of the bladder, the clinician may use the information concerning the levels or presence of the said Pax 5 to facilitate decision making regarding treatment of the patient. Thus, if the level (for example below the limit of detection) of Pax 5 is indicative of a low invasive potential of said bladder cancer, unnecessary surgery may be avoided. Similarly, if the level (for example a detectable level) of Pax 5 is

indicative of a high invasive potential of said bladder cancer, surgery may be the preferred treatment. Thus, cystectomy (total removal of the bladder) may be indicated by the detection of Pax 5 expression, for example with consideration of other clinical factors, as discussed above.

Possible treatments for bladder cancer include resection of the tumour from inside the bladder (TURBT), total removal of the bladder (cystectomy), radiotherapy or local or systemic chemotherapy. It will be appreciated that treatments may be used in combination.

It will be appreciated from the foregoing, and from the Examples below, that the determination of the levels (including detectable presence/absence) of the said Pax 5 may be exploited diagnostically to predict whether a given bladder cancer, particularly transitional cell carcinoma of the bladder, would invade surrounding tissue or metastasise, since expression of said Pax 5 is believed to correspond to possible future invasion and/or spread of a tumour. Determination of the levels of Pax 5 may be useful in monitoring the recurrence of bladder cancer in patients who have received treatment for bladder cancer, for example treatment which appears to have removed the bladder cancer cells.

It will be appreciated that reference to determining the level of Pax 5 expression includes the meaning of determining whether Pax 5 expression may be detected or not.

It is also particularly preferred if the method of the invention is employed to predict whether a given bladder cancer would invade surrounding tissue.

In one preferred embodiment of the invention it is determined whether the level of said Pax 5 nucleic acid, in particular mRNA, is a level associated with cancer. Preferably, the sample contains nucleic acid, such as mRNA, and the level of said Pax 5 is measured by contacting said nucleic acid with a nucleic acid which hybridises selectively to said Pax 5 nucleic acid.

By "selectively hybridising" is meant that the nucleic acid has sufficient nucleotide sequence similarity with the said human nucleic acid that it can hybridise under moderately or highly stringent conditions, and preferably does not hybridise to other Pax nucleic acids under the same conditions. As is well known in the art, the stringency of nucleic acid hybridization depends on factors such as length of nucleic acid over which hybridisation occurs, degree of identity of the hybridising sequences and on factors such as temperature, ionic strength and CG or AT content of the sequence. Thus, any nucleic acid which is capable of selectively hybridising as said is useful in the practice of the invention.

Nucleic acids which can selectively hybridise to the said human nucleic acid include nucleic acids which have >95% sequence identity, preferably those with >98%, more preferably those with >99% sequence identity, over at least a portion of the nucleic acid with the said human nucleic acid. As is well known, human genes usually contain introns such that, for example, a mRNA or cDNA derived from a gene would not match perfectly along its entire length with the said human genomic DNA but would nevertheless be a nucleic acid capable of selectively hybridising to the said human DNA. Thus, the invention specifically includes nucleic acids which selectively hybridise to said Pax 5 mRNA or cDNA but may not hybridise to a said Pax 5 gene. For example, nucleic acids which span

the intron-exon boundaries of the said Pax 5 gene may not be able to selectively hybridise to the said Pax 5 mRNA or cDNA.

Typical moderately or highly stringent hybridisation conditions which lead to selective hybridisation are known in the art, for example those described in *Molecular Cloning, a laboratory manual*, 2nd edition, Sambrook *et al* (eds), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, incorporated herein by reference.

An example of a typical hybridisation solution when a nucleic acid is immobilised on a nylon membrane and the probe nucleic acid is ≥ 500 bases is:

6 x SSC (saline sodium citrate)
0.5% sodium dodecyl sulphate (SDS)
100 µg/ml denatured, fragmented salmon sperm DNA

The hybridisation is performed at 68°C. The nylon membrane, with the nucleic acid immobilised, may be washed at 68°C in 1 x SSC or, for high stringency, 0.1 x SSC.

20 x SSC may be prepared in the following way. Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml of H_2O. Adjust the pH to 7.0 with a few drops of a 10 N solution of NaOH. Adjust the volume to 1 litre with H_2O. Dispense into aliquots. Sterilise by autoclaving.

An example of a typical hybridisation solution when a nucleic acid is immobilised on a nylon membrane and the probe is an oligonucleotide of between 15 and 50 bases is:

3.0 M trimethylammonium chloride (TMACl)

0.01 M sodium phosphate (pH 6.8)

1 mm EDTA (pH 7.6)

0.5% SDS

100 μg/ml denatured, fragmented salmon sperm DNA

0.1% non-fat dried milk

The optimal temperature for hybridization is usually chosen to be 5°C below the T_i for the given chain length. T_i is the irreversible melting temperature of the hybrid formed between the probe and its target sequence. Jacobs *et al* (1988) *Nucl. Acids Res.* **16**, 4637 discusses the determination of T_is. The recommended hybridization temperature for 17-mers in 3 M TMACl is 48-50°C; for 19-mers, it is 55-57°C; and for 20-mers, it is 58-66°C.

By "nucleic acid which selectively hybridises" is also included nucleic acids which may be used to amplify DNA from the Pax 5 cDNA (for example formed by reverse transcription of mRNA) by any of the well known amplification systems such as those described in more detail below, in particular the polymerase chain reaction (PCR).

It is preferred that PCR is used in the methods of the invention.

Suitable conditions for PCR amplification include amplification in a suitable 1 x amplification buffer:

10 x amplification buffer is 500 mM KCl; 100 mM Tris.Cl (pH 8.3 at room temperature); 15 mM $MgCl_2$; 0.1% gelatin.

A suitable denaturing agent or procedure (such as heating to 95°C) is used in order to separate the strands of double-stranded DNA.

Suitably, the annealing part of the amplification is between 37°C and 65°C. The optimum temperature may be 50°C for most primer pairs, but as discussed in Example 1, a temperature of 65 °C may be preferred for the Pax 5 amplification primers shown below:

Upstream primer: 5' AGGATGCCGCTGATGGAGTAC 3'
Downstream primer: 5' TGGAGGAGTGAATCAGCTTGG 3'

An annealing temperature of 55 °C may be preferred for the GAPDH amplification primers shown below:

Upstream primer: 5' GGCCGTATTGGGCGCCTGGTC 3'
Downstream primer: 5' GAAGGGCAACTACTGTTCGAAG 3'
or
Upstream primer 5'ACTGGCGTCTTCACCACCATG3'
Downstream primer 5'GTCATGGATGACCTTGGCCAG3'.

These primers may be used in an amplification reaction alongside a reaction using primers, such as those described above, that may amplify nucleic acid encoding part of Pax 5, such that the GAPDH amplification that serves as a positive control for the detection of nucleic acid, as described below, particularly in Example 1.

A temperature of 72 °C may be used for the extension phase of the amplification when a thermostable polymerase is used, such as *Taq* polymerase.

Although the nucleic acid which is useful in the methods of the invention may be RNA or DNA, DNA is preferred. Although the nucleic acid which is useful in the methods of the invention may be double-stranded or single-stranded, single-stranded nucleic acid is preferred under some circumstances such as in nucleic acid amplification reactions.

The nucleic acid which is useful in the methods of the invention may be any suitable size. However, for certain diagnostic, probing or amplifying purposes, it is preferred if the nucleic acid has fewer than 10 000, more preferably fewer than 1000, more preferably still from 10 to 100, and in further preference from 15 to 30 base pairs (if the nucleic acid is double-stranded) or bases (if the nucleic acid is single stranded). As is described more fully below, single-stranded DNA primers, suitable for use in a polymerase chain reaction, are particularly preferred.

The nucleic acid for use in the methods of the invention is a nucleic acid capable of hybridising to the said Pax 5 mRNAs. Fragments of the said Pax 5 genes and cDNAs derivable from the mRNA encoded by the said Pax 5 genes are also preferred nucleic acids for use in the methods of the invention.

It is particularly preferred if the nucleic acid for use in the methods of the invention is an oligonucleotide primer which can be used to amplify a portion of the said Pax 5 nucleic acid, particularly Pax 5 mRNA. Examples are the primers described above:

Upstream primer: 5' AGGATGCCGCTGATGGAGTAC 3'

Downstream primer: 5' TGGAGGAGTGAATCAGCTTGG 3'

The Pax 5 mRNA is similar to, but distinct from other Pax mRNAs. Preferred nucleic acids for use in the invention are those that selectively hybridise to the Pax 5 mRNA and do not hybridise to other Pax 5 mRNAs. Such selectively hybridising nucleic acids can be readily obtained, for example, by reference to whether or not they hybridise to the said Pax 5 mRNA or cDNA and not to other Pax mRNAs or cDNAs. The paired box domain is highly conserved, but the carboxy terminal end is variable, so it may be preferred that a selectively hybridising nucleic acid hybridises to a part of a nucleic acid that does not encode the paired box domain, and preferably encodes part of the carboxy terminal region of Pax 5, excluding the paired box domain.

The methods are suitable in respect of any bladder cancer but it is preferred if the cancer is transitional cell carcinoma of the bladder.

It is preferred if the nucleic acid is derived from a sample of the tissue in which cancer is suspected or in which cancer may be or has been found. It is preferred if the sample containing nucleic acid is derived from the bladder or urethra, particularly the urothelium, of the patient. Samples of bladder, urethra or urothelium may be obtained by surgical excision, laproscopy and biopsy, endoscopy and biopsy, flexible cytoscopy and image-guided biopsy. The image may be generated by ultrasound or technetium-99-labelled antibodies or antibody fragments which bind or locate selectively to the urothelium.

It is preferred if the sample is selected from the group consisting of urothelium or urine. Urothelium can be obtained from a patient using standard surgical techniques. Both normal and malignant urothelium sheds cells into the urine, and therefore cells derived from the urothelium may be found in small numbers in the urine. Although it is preferred that the sample containing nucleic acid from the patient is, or is derived directly from, a cell of the patient, such as a urothelial cell, a sample indirectly derived from a patient, such as a cell grown in culture, is also included within the invention. Equally, although the nucleic acid derived from the patient may have been physically within the patient, it may alternatively have been copied from nucleic acid which was physically within the patient. The tumour tissue may be taken from the primary tumour or from metastases, and particularly may be taken from the margins of the tumour. Malignant cells may be present in the blood (micrometastases) and therefore blood samples or bladder cancer cells separated from the blood may be used as a sample.

It will be appreciated that the aforementioned methods may be used for presymptomatic screening of a patient who is in a risk group for bladder cancer, as discussed above. For example, persons older than about 40 to 50 years are at greater risk of bladder cancer than persons below the age of 35; most patients are between 50 and 70 years of age.. Men may be at greater risk than women. Smokers and workers in the dye (aniline), rubber and leather industries may also be at greater risk. Similarly, the methods may be used for the pathological classification of tumours such as bladder cancer tumours.

It is preferred that if semen or urine is the source of the said sample containing nucleic acid derived from the patient that the sample is enriched

for urothelium-derived tissue or cells. Enrichment for urothelial cells may be achieved using, for example, cell sorting methods such as fluorescent activated cell sorting (FACS) using a urothelium-selective antibody. The source of the said sample also includes biopsy material and tumour samples, also including fixed paraffin mounted specimens as well as fresh or frozen tissue. It is preferred that tissue is frozen at -70 °C as rapidly as possible after removal from the patient, in order to minimise degradation of mRNA in the tissue prior to any extraction of the mRNA.

Conveniently, the nucleic acid capable of selectively hybridising to the said human nucleic acid such as mRNA and which is used in the methods of the invention further comprises a detectable label.

By "detectable label" is included any convenient radioactive label such as ^{32}P, ^{33}P or ^{35}S which can readily be incorporated into a nucleic acid molecule using well known methods; any convenient fluorescent or chemiluminescent label which can readily be incorporated into a nucleic acid is also included. In addition the term "detectable label" also includes a moiety which can be detected by virtue of binding to another moiety (such as biotin which can be detected by binding to streptavidin); and a moiety, such as an enzyme, which can be detected by virtue of its ability to convert a colourless compound into a coloured compound, or *vice versa* (for example, alkaline phosphatase can convert colourless o-nitrophenylphosphate into coloured *o*-nitrophenol). Conveniently, the nucleic acid probe may occupy a certain position in a fixed array and whether the nucleic acid hybridises to the said Pax 5 nucleic acid can be determined by reference to the position of hybridisation in the fixed array.

Labelling with [^{32}P]dCTP may be carried out using a Rediprime® random primer labelling kit supplied by Amersham.

Primers which are suitable for use in a polymerase chain reaction (PCR; Saiki *et al* (1988) *Science* **239**, 487-491) are preferred. Suitable PCR primers may have the following properties:

It is well known that the sequence at the 5′ end of the oligonucleotide need not match the target sequence to be amplified.

It is usual that the PCR primers do not contain any complementary structures with each other longer than 2 bases, especially at their 3′ ends, as this feature may promote the formation of an artefactual product called "primer dimer". When the 3′ ends of the two primers hybridise, they form a "primed template" complex, and primer extension results in a short duplex product called "primer dimer".

Internal secondary structure should be avoided in primers. For symmetric PCR, a 40-60% G+C content is often recommended for both primers, with no long stretches of any one base. The classical melting temperature calculations used in conjunction with DNA probe hybridization studies often predict that a given primer should anneal at a specific temperature or that the 72°C extension temperature will dissociate the primer/template hybrid prematurely. In practice, the hybrids are more effective in the PCR process than generally predicted by simple T_m calculations.

Optimum annealing temperatures may be determined empirically and may be higher than predicted. *Taq* DNA polymerase does have activity in the 37-55°C region, so primer extension will occur during the annealing step

and the hybrid will be stabilised. The concentrations of the primers are equal in conventional (symmetric) PCR and, typically, within 0.1- to 1-μM range.

It will be appreciated that it may be beneficial to choose primers that will yield different size products from genomic and mRNA-derived templates, for example primers that lie within different exons, so that product amplified from genomic DNA will include one or more intron sequences, whereas product amplified from mRNA-derived template will not include intron sequences.

It will further be appreciated that if a control amplification reaction is to be carried out, for example using primers complementary to an ubiquitously expressed protein, for example GAPDH, that it may be beneficial for the products of the control and Pax 5 derived products to be of different sizes, such that the two products may be distinguished by the detection means employed, for example by mobility on agarose gel electrophoresis. However, it may be desirable for the two products to be of similar size, for example both between 100 and 1000, or between 100 and 600 nucleotides long. This may aid simultaneous analysis of the products, for example by gel electrophoresis, and may also mean that the control and Pax 5 amplification reactions may have similar performance characteristics, in terms, for example, of relative rates of accumulation of product at different stages during the reaction.

Any of the nucleic acid amplification protocols can be used in the method of the invention including the polymerase chain reaction, QB replicase and ligase chain reaction. Also, NASBA (nucleic acid sequence based amplification), also called 3SR, can be used as described in Compton

(1991) *Nature* **350**, 91-92 and *AIDS* (1993), Vol 7 (Suppl 2), S108 or SDA (strand displacement amplification) can be used as described in Walker *et al* (1992) *Nucl. Acids Res.* **20**, 1691-1696. The polymerase chain reaction is particularly preferred because of its simplicity.

When a pair of suitable nucleic acids of the invention are used in a PCR it is convenient to detect the product by gel electrophoresis and ethidium bromide staining. As an alternative to detecting the product of DNA amplification using agarose gel electrophoresis and ethidium bromide staining of the DNA, it is convenient to use a labelled oligonucleotide capable of hybridising to the amplified DNA as a probe. When the amplification is by a PCR the oligonucleotide probe hybridises to the interprimer sequence as defined by the two primers. The oligonucleotide probe is preferably between 10 and 50 nucleotides long, more preferably between 15 and 30 nucleotides long. It may be longer than the amplified DNA or include one or both of the primers, but in this case, the hybridisation conditions should be such that the probe should not hybridise to the primers alone, but only to an amplified product that also contains interprimer sequence that is capable of hybridising to the probe.

The probe may be labelled with a radionuclide such as ^{32}P, ^{33}P and ^{35}S using standard techniques, or may be labelled with a fluorescent dye. When the oligonucleotide probe is fluorescently labelled, the amplified DNA product may be detected in solution (see for example Balaguer *et al* (1991) "Quantification of DNA sequences obtained by polymerase chain reaction using a bioluminescence adsorbent" *Anal. Biochem.* **195**, 105-110 and Dilesare *et al* (1993) "A high-sensitivity electrochemiluminescence-based detection system for automated PCR product quantitation" *BioTechniques* **15**, 152-157.

PCR products can also be detected using a probe which may have a fluorophore-quencher pair or may be attached to a solid support or may have a biotin tag or they may be detected using a combination of a capture probe and a detector probe.

Fluorophore-quencher pairs are particularly suited to quantitative measurements of PCR reactions (eg RT-PCR). Fluorescence polarisation using a suitable probe may also be used to detect PCR products.

Thus, in a preferred method of the invention, total RNA may be prepared from a patient sample or a control sample (as discussed above) using TRIZOL® reagent (Life Technologies ™), according to the manufacturers' instructions and 5 μg of total RNA from each sample may be reverse transcribed using a first-strand cDNA synthesis kit (Pharmacia - protocol according to manufacturers' instructions). PCR amplification for Pax 5 cDNA may be performed on the RT product. The ubiquitiously expressed GAPDH may be amplified as a control. For amplification of Pax 5 cDNA, the protocol may consist of 35 cycles of denaturation at 95 °C, annealing at 65 °C and extension at 72 °C. For GAPDH, the annealing temperature may be 55 °C. PCR primers may be designed from the published sequence of Pax 5 (13). For example, primers used and the size of PCR products may be as follows; Pax 5 (500 base pairs), upstream: 5'AGGATGCCGCTGATGGAGTAC 3' and downstream: 5' TGGAGGAGTGAATCAGCTTGG 3'. GAPDH (190 base pairs), upstream: 5'GGCCGTATTGGCGCCTGGTC 3' and downstream 5' GAAGGGCAACTACTGTTCGAAG 3' or upstream 5'ACTGGCGTCTTCACCACCATG3' with downstream

5'GTCATGGATGACCTTGGCCAG3' (194 base pairs). Negative controls may be included with water replacing cDNA.

The above primers are designed so that each anneals within an exon, but the amplified fragment crosses boundaries between exons, so that inadvertent amplification of genomic DNA would include an intron. This would easily be identified by the larger size of the fragment on the gel. Positive controls used may be B-lymphoblastoid cells known to express Pax 5. The B-lymphoblast cell line WEHI-231 may be used as a positive control for Pax 5 expression, as described in Example 1. For the Pax 5 and at least the second set of GAPDH primers shown above, at least two introns are included between the primers if genomic DNA is amplified.

PCR products may electrophoresed on 1.8% agarose gels and transferred to nylon membranes (Qiabrane Nylon Plus, Qiagen). Filters may hybridised with a human Pax 5 cDNA probe designed from the Pax 5 cDNA sequence, for example the 1.2 kb human Pax 5 cDNA probe 101691 from the Max Planck Institut, Goettingen, labeled with [^{32}P]dCTP using a Rediprime® random primer labelling kit (Amersham) and ExpressHybe® (Clontech) according to the manufacturers' instructions. The filters may then be exposed to x-ray film. A 20 minute exposure of the filters to the film may be sufficient for detection of the control Pax 5-containing sample and of Pax 5 positive samples. Control GAPDH-product may be detected in an analagous manner, or by ethidium bromide detection on the gel prior to blotting or on a duplicate gel, as described below.

Alternatively, both Pax 5 and control, for example GAPDH, products may be detected after agarose gel electrophoresis by ethidium bromide staining

and UV detection of the products, as well known to those skilled in the art. Ethidium bromide may be added to 100μg per 100 ml gel (ie 100μl of 1mg/ml ethidium bromide solution to 100 ml of agarose solution).

Thus, using the above method, Pax 5 expression may be detected in bladder cancer tissue, particularly from invasive bladder cancer, but may not be detected in normal bladder tissue, whereas GAPDH expression may be detected in bladder cancer and normal bladder tissue.

It will be appreciated that the greater frequency of detection of Pax 5 expression in tumours of higher grade may indicate higher levels of expression of Pax 5 mRNA in tumours of higher grade and therefore a higher probability of the Pax 5 mRNA being amplified to a level at which the amplification product may be detected by the methods described above.

In a further preferred embodiment, the level of said Pax 5 protein is measured. Preferably, the level of said protein is measured by contacting the protein with a molecule which selectively binds to Pax 5

The sample containing protein derived from the patient is conveniently a sample tissue.

The sample containing protein derived from the patient is conveniently a sample of the tissue in which cancer is suspected or in which cancer may be or has been found. These methods may be used for any bladder cancer, but they are particularly suitable in respect of transitional cell carcinoma of the bladder. Methods of obtaining suitable samples are described in relation to earlier methods.

The methods of the invention involving detection of the said Pax 5 proteins are particularly useful in relation to historical samples such as those containing paraffin-embedded sections of tumour samples.

The level of said Pax 5 protein may be determined in a sample in any suitable way.

It is particularly preferred if the molecule which selectively binds to Pax 5 is an antibody.

Antibodies which can selectively bind to a particular form of Pax 5 can be made, for example, by using peptides which encompass the differences between Pax 5 and other Pax proteins.

The antibodies may be monoclonal or polyclonal. Suitable monoclonal antibodies may be prepared by known techniques, for example those disclosed in "Monoclonal Antibodies: A manual of techniques", H Zola (CRC Press, 1988) and in "Monoclonal Hybridoma Antibodies: Techniques and applications", J G R Hurrell (CRC Press, 1982), both of which are incorporated herein by reference.

Polyclonal antibodies to Pax 5 may be obtained from Santa Cruz Biotech. Pax 5 (C-20), cat # sc-1974, is a goat polyclonal IgG, reacting with the epitope corresponding to amino acids 371-390 at the carboxy terminus of Pax 5. Pax 5 (N-19), cat # sc-1975, is a goat polyclonal IgG, reacting with the epitope corresponding to amino acids 2 to 20 at the amino terminus of Pax 5. These antibodies are described as not cross-reactive with other paired box transcription factors, and are suitable for use in

Western blotting and immunohistochemistry. The antibodies are also supplied by Santa Cruz Biotech as reagents suitable for use in gel-shift assays of Pax 5 binding to target nucleic acid.

By "the relative amount of said Pax 5 protein" is meant the amount of said Pax 5 protein per unit mass of sample tissue or per unit number of sample cells compared to the amount of said Pax 5 protein per unit mass of known normal tissue or per unit number of normal cells. The relative amount may be determined using any suitable protein quantitation method. In particular, it is preferred if antibodies are used and that the amount of said Pax 5 protein is determined using methods which include quantitative western blotting, enzyme-linked immunosorbent assays (ELISA) or quantitative immunohistochemistry.

Other techniques for raising and purifying antibodies are well known in the art and any such techniques may be chosen to achieve the preparations useful in the methods claimed in this invention. In a preferred embodiment of the invention, antibodies will immunoprecipitate said Pax 5 proteins from solution as well as react with said Pax 5 protein on western or immunoblots of polyacrylamide gels. In another preferred embodiment, antibodies will detect said Pax 5 proteins in paraffin or frozen tissue sections, or in cells recovered from urine samples, using immunocytochemical techniques. For example, a method involving FITC immunofluorescence may conveniently be used.

Preferred embodiments relating to methods for detecting said Pax 5 protein include enzyme linked immunosorbent assays (ELISA), radioimmunoassay (RIA), immunoradiometric assays (IRMA) and immunoenzymatic assays (IEMA), including sandwich assays using

monoclonal and/or polyclonal antibodies. Exemplary sandwich assays are described by David *et al* in US Patent Nos. 4,376,110 and 4,486,530, hereby incorporated by reference. Antibody staining of cells on slides, for example cells recovered from urine samples, may be used, using antibodies to Pax 5 in methods well known in cytology laboratory diagnostic tests, as well known to those skilled in the art.

It will be appreciated that other antibody-like molecules may be used in the method of the inventions including, for example, antibody fragments or derivatives which retain their antigen-binding sites, synthetic antibody-like molecules such as single-chain Fv fragments (ScFv) and domain antibodies (dAbs), and other molecules with antibody-like antigen binding motifs.

In a further embodiment the level of Pax 5 is measured by selectively assaying its activity in the sample. Thus, the binding of Pax 5 to its target DNA sequence may be measured. This may be done using gel-shift assays, for example as described in Adams *et al* (1992) *Genes & Dev* 6, 1589-1607.

A further aspect of the invention provides use of an agent which is capable of use in determining the level of Pax 5 protein or nucleic acid in a sample in the manufacture of a reagent for diagnosing bladder cancer. The agent may suitably be a nucleic acid which selectively hybridises to Pax 5 nucleic acid or the agent may be a molecule which selectively binds to Pax 5 protein or the agent may be an agent useful in selectively assaying the activity of Pax 5. The agents as defined are therefore useful in a method of diagnosing bladder cancer or non-malignant pathological processes of the urothelium, particularly transitional cell carcinoma of the bladder.

A further aspect of the invention comprises a kit of parts useful for diagnosing bladder cancer, especially transitional cell carcinoma of the bladder, comprising an agent which is capable of use in determining the level of Pax 5 protein or nucleic acid in a sample. The agent may be a nucleic acid which selectively hybridises to Pax 5 nucleic acid or the agent may be a molecule which selectively binds to Pax 5 protein or the agent may be an agent useful in selectively assaying the activity of Pax 5.

Preferably, the kit further comprises a control sample containing Pax 5 nucleic acid or protein wherein the control sample may be a negative control (which contains a level of Pax 5 protein or nucleic acid which is not associated with cancer or a high invasive potential for bladder cancer particularly transitional cell carcinoma of the bladder, for example a level of Pax 5 protein or nucleic acid which is not detectable) or it may be a positive control (which contains a level of Pax 5 protein or nucleic acid which is associated with bladder cancer or a high invasive potential for bladder cancer, particularly transitional cell carcinoma of the bladder, for example a level that may be detected). The kit may contain both negative and positive controls. The kit may usefully contain controls of Pax 5 protein or nucleic acid which correspond to different amounts such that a calibration curve may be made.

A further aspect of the invention provides a method of treating bladder cancer comprising the step of administering to the patient an agent which selectively prevents the function of Pax 5.

By "an agent which selectively prevents the function of Pax 5" we include agents that (a) inhibit the expression of said Pax 5 or (b) inhibit the activity of said Pax 5.

Agents that prevent the expression of said Pax 5 include but are not limited to antisense agents. Retinoic acid may inhibit Pax 5 activity.

Antisense oligonucleotides are single-stranded nucleic acid, which can specifically bind to a complementary nucleic acid sequence. By binding to the appropriate target sequence, an RNA-RNA, a DNA-DNA, or RNA-DNA duplex is formed. These nucleic acids are often termed "antisense" because they are complementary to the sense or coding strand of the gene. Recently, formation of a triple helix has proven possible where the oligonucleotide is bound to a DNA duplex. It was found that oligonucleotides could recognise sequences in the major groove of the DNA double helix. A triple helix was formed thereby. This suggests that it is possible to synthesise a sequence-specific molecules which specifically bind double-stranded DNA *via* recognition of major groove hydrogen binding sites.

By binding to the target nucleic acid, the above oligonucleotides can inhibit the function of the target nucleic acid. This could, for example, be a result of blocking the transcription, processing, poly(A)addition, replication, translation, or promoting inhibitory mechanisms of the cells, such as promoting RNA degradations.

Antisense oligonucleotides are prepared in the laboratory and then introduced into cells, for example by microinjection or uptake from the cell culture medium into the cells, or they are expressed in cells after

transfection with plasmids or retroviruses or other vectors carrying an antisense gene. Antisense oligonucleotides were first discovered to inhibit viral replication or expression in cell culture for Rous sarcoma virus, vesicular stomatitis virus, herpes simplex virus type 1, simian virus and influenza virus. Since then, inhibition of mRNA translation by antisense oligonucleotides has been studied extensively in cell-free systems including rabbit reticulocyte lysates and wheat germ extracts. Inhibition of viral function by antisense oligonucleotides has been demonstrated *in vitro* using oligonucleotides which were complementary to the AIDS HIV retrovirus RNA (Goodchild, J. 1988 "Inhibition of Human Immunodeficiency Virus Replication by Antisense Oligodeoxynucleotides", *Proc. Natl. Acad. Sci. (USA)* 85(15), 5507-11). The Goodchild study showed that oligonucleotides that were most effective were complementary to the poly(A) signal; also effective were those targeted at the 5′ end of the RNA, particularly the cap and 5′ untranslated region, next to the primer binding site and at the primer binding site. The cap, 5′ untranslated region, and poly(A) signal lie within the sequence repeated at the ends of retrovirus RNA (R region) and the oligonucleotides complementary to these may bind twice to the RNA.

Oligonucleotides are subject to being degraded or inactivated by cellular endogenous nucleases. To counter this problem, it is possible to use modified oligonucleotides, eg having altered internucleotide linkages, in which the naturally occurring phosphodiester linkages have been replaced with another linkage. For example, Agrawal *et al* (1988) *Proc. Natl. Acad. Sci. USA* 85, 7079-7083 showed increased inhibition in tissue culture of HIV-1 using oligonucleotide phosphoramidates and phosphorothioates. Sarin *et al* (1988) *Proc. Natl. Acad. Sci. USA* 85, 7448-7451 demonstrated increased inhibition of HIV-1 using oligonucleotide methylphosphonates.

Agrawal *et al* (1989) *Proc. Natl. Acad. Sci. USA* **86**, 7790-7794 showed inhibition of HIV-1 replication in both early-infected and chronically infected cell cultures, using nucleotide sequence-specific oligonucleotide phosphorothioates. Leither *et al* (1990) *Proc. Natl. Acad. Sci. USA* **87**, 3430-3434 report inhibition in tissue culture of influenza virus replication by oligonucleotide phosphorothioates.

Oligonucleotides having artificial linkages have been shown to be resistant to degradation *in vivo*. For example, Shaw *et al* (1991) in *Nucleic Acids Res.* **19**, 747-750, report that otherwise unmodified oligonucleotides become more resistant to nucleases *in vivo* when they are blocked at the 3′ end by certain capping structures and that uncapped oligonucleotide phosphorothioates are not degraded *in vivo*.

A detailed description of the H-phosphonate approach to synthesising oligonucleoside phosphorothioates is provided in Agrawal and Tang (1990) *Tetrahedron Letters* **31**, 7541-7544, the teachings of which are hereby incorporated herein by reference. Syntheses of oligonucleoside methylphosphonates, phosphorodithioates, phosphoramidates, phosphate esters, bridged phosphoramidates and bridge phosphorothioates are known in the art. See, for example, Agrawal and Goodchild (1987) *Tetrahedron Letters* **28**, 3539; Nielsen *et al* (1988) *Tetrahedron Letters* **29**, 2911; Jager *et al* (1988) *Biochemistry* **27**, 7237; Uznanski *et al* (1987) *Tetrahedron Letters* **28**, 3401; Bannwarth (1988) *Helv. Chim. Acta.* **71**, 1517; Crosstick and Vyle (1989) *Tetrahedron Letters* **30**, 4693; Agrawal *et al* (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1401-1405, the teachings of which are incorporated herein by reference. Other methods for synthesis or production also are possible. In a preferred embodiment the oligonucleotide

is a deoxyribonucleic acid (DNA), although ribonucleic acid (RNA) sequences may also be synthesised and applied.

The oligonucleotides useful in the invention preferably are designed to resist degradation by endogenous nucleolytic enzymes. *In vivo* degradation of oligonucleotides produces oligonucleotide breakdown products of reduced length. Such breakdown products are more likely to engage in non-specific hybridization and are less likely to be effective, relative to their full-length counterparts. Thus, it is desirable to use oligonucleotides that are resistant to degradation in the body and which are able to reach the targeted cells. The present oligonucleotides can be rendered more resistant to degradation *in vivo* by substituting one or more internal artificial internucleotide linkages for the native phosphodiester linkages, for example, by replacing phosphate with sulphur in the linkage. Examples of linkages that may be used include phosphorothioates, methylphosphonates, sulphone, sulphate, ketyl, phosphorodithioates, various phosphoramidates, phosphate esters, bridged phosphorothioates and bridged phosphoramidates. Such examples are illustrative, rather than limiting, since other internucleotide linkages are known in the art. See, for example, Cohen, (1990) *Trends in Biotechnology*. The synthesis of oligonucleotides having one or more of these linkages substituted for the phosphodiester internucleotide linkages is well known in the art, including synthetic pathways for producing oligonucleotides having mixed internucleotide linkages.

Oligonucleotides can be made resistant to extension by endogenous enzymes by "capping" or incorporating similar groups on the 5′ or 3′ terminal nucleotides. A reagent for capping is commercially available as Amino-Link II™ from Applied BioSystems Inc, Foster City, CA. Methods for capping are described, for example, by Shaw *et al* (1991) *Nucleic Acids*

Res. 19, 747-750 and Agrawal *et al* (1991) *Proc. Natl. Acad. Sci. USA* 88(17), 7595-7599, the teachings of which are hereby incorporated herein by reference.

A further method of making oligonucleotides resistant to nuclease attack is for them to be "self-stabilised" as described by Tang *et al* (1993) *Nucl. Acids Res.* 21, 2729-2735 incorporated herein by reference. Self-stabilised oligonucleotides have hairpin loop structures at their 3' ends, and show increased resistance to degradation by snake venom phosphodiesterase, DNA polymerase I and fetal bovine serum. The self-stabilised region of the oligonucleotide does not interfere in hybridization with complementary nucleic acids, and pharmacokinetic and stability studies in mice have shown increased *in vivo* persistence of self-stabilised oligonucleotides with respect to their linear counterparts.

It is preferred that the antisense reagent is able to bind to Pax 5 nucleic acid, but not nucleic acid encoding any other Pax protein. It may therefore be preferred that the antisense reagent does not hybridise to the region of the Pax 5 nucleic acid encoding the paired box domain, but may hybridise to the carboxy terminal region of Pax 5.

In accordance with the invention, the antisense compound may be administered systemically. Alternatively, the inherent binding specificity of antisense oligonucleotides characteristic of base pairing may be enhanced by limiting the availability of the antisense compound to its intend locus *in vivo*, permitting lower dosages to be used and minimising systemic effects. Thus, oligonucleotides may be applied locally to achieve the desired effect. The concentration of the oligonucleotides at the desired locus is much higher than if the oligonucleotides were administered systemically, and the

therapeutic effect can be achieved using a significantly lower total amount. The local high concentration of oligonucleotides enhances penetration of the targeted cells and effectively blocks translation of the target nucleic acid sequences.

The oligonucleotides can be delivered to the locus by any means appropriate for localised administration of a drug. For example, a solution of the oligonucleotides can be injected directly to the site or can be delivered by infusion using an infusion pump. The oligonucleotides also can be incorporated into an implantable device which when placed at the desired site, permits the oligonucleotides to be released into the surrounding locus. Thus, oligonucleotides may be delivered by a method analagous to the technique of intravesicle chemotherapy known to those skilled in the art, in which chemotherapeutic agents are introduced into the bladder through a catheter installed under local anaesthetic, following which the patient may move or roll around for approximately two hours in order to distribute the agent around the bladder.

The oligonucleotides may be administered *via* a hydrogel material. The hydrogel is noninflammatory and biodegradable. Many such materials now are known, including those made from natural and synthetic polymers. In a preferred embodiment, the method exploits a hydrogel which is liquid below body temperature but gels to form a shape-retaining semisolid hydrogel at or near body temperature. Preferred hydrogel are polymers of ethylene oxide-propylene oxide repeating units. The properties of the polymer are dependent on the molecular weight of the polymer and the relative percentage of polyethylene oxide and polypropylene oxide in the polymer. Preferred hydrogels contain from about 10 to about 80% by weight ethylene oxide and from about 20 to about 90% by weight propylene oxide. A

particularly preferred hydrogel contains about 70% polyethylene oxide and 30% polypropylene oxide. Hydrogels which can be used are available, for example, from BASF Corp., Parsippany, NJ, under the tradename Pluronic^{R}.

In this embodiment, the hydrogel is cooled to a liquid state and the oligonucleotides are admixed into the liquid to a concentration of about 1 mg oligonucleotide per gram of hydrogel. The resulting mixture then is applied onto the surface to be treated, for example by spraying or painting during surgery or using a catheter or endoscopic procedures. As the polymer warms, it solidifies to form a gel, and the oligonucleotides diffuse out of the gel into the surrounding cells over a period of time defined by the exact composition of the gel. The hydrogel may be administered intravesically, as described above.

It will be appreciated that the oligonucleotides or other agents may be administered after surgical removal of a tumour, and may be administered to the area from which the tumour has been removed, and surrounding tissue, for example using cytoscopy to guide application of the oligonucleotides or other agents.

The oligonucleotides can be administered by means of other implants that are commercially available or described in the scientific literature, including liposomes, microcapsules and implantable devices. For example, implants made of biodegradable materials such as polyanhydrides, polyorthoesters, polylactic acid and polyglycolic acid and copolymers thereof, collagen, and protein polymers, or non-biodegradable materials such as ethylenevinyl acetate (EVAc), polyvinyl acetate, ethylene vinyl alcohol, and derivatives thereof can be used to locally deliver the oligonucleotides. The

oligonucleotides can be incorporated into the material as it is polymerised or solidified, using melt or solvent evaporation techniques, or mechanically mixed with the material. In one embodiment, the oligonucleotides are mixed into or applied onto coatings for implantable devices such as dextran coated silica beads, stents, or catheters.

The dose of oligonucleotides is dependent on the size of the oligonucleotides and the purpose for which is it administered. In general, the range is calculated based on the surface area of tissue to be treated. The effective dose of oligonucleotide is somewhat dependent on the length and chemical composition of the oligonucleotide but is generally in the range of about 30 to 3000 μg per square centimetre of tissue surface area.

The oligonucleotides may be administered to the patient systemically for both therapeutic and prophylactic purposes. The oligonucleotides may be administered by any effective method, for example, parenterally (eg intravenously, subcutaneously, intramuscularly) or by oral, nasal or other means which permit the oligonucleotides to access and circulate in the patient's bloodstream. Oligonucleotides administered systemically preferably are given in addition to locally administered oligonucleotides, but also have utility in the absence of local administration. A dosage in the range of from about 0.1 to about 10 grams per administration to an adult human generally will be effective for this purpose.

It will be appreciated that it may be desirable to target the antisense oligonucleotides to the bladder. This may be achieved by administering the antisense oligonucleotides to the bladder, for example as described above, or it may be achieved by using antisense oligonucleotides which are in association with a molecule which selectively directs the antisense

oligonucleotide to the bladder. For example, the antisense oligonucleotide may be associated with an antibody or antibody like molecule which selectively binds a bladder-related antigen. Such antigens are well known to those skilled in the art. By "associated with" we mean that the antisense oligonucleotide and the bladder-directing entity are so associated that the bladder-directing entity is able to direct the antisense oligonucleotide to the bladder cells, for example urothelial cells.

It will be appreciated that antisense agents also include larger molecules which bind to said Pax 5 mRNA or genes and substantially prevent expression of said Pax 5 mRNA or genes and substantially prevent expression of said Pax 5 protein. Thus, expression of an antisense molecule which is substantially complementary to said Pax 5 mRNA is envisaged as part of the invention.

As discussed above, it is preferred that the antisense molecule is not able to substantially prevent expression of other Pax mRNAs or proteins. It may therefore preferably not be complementary solely to the region of the Pax 5 mRNA that encodes the paired box domain, and is more preferably not complementary to the region of the Pax 5 mRNA that encodes the paired box domain.

The said larger molecules may be expressed from any suitable genetic construct as is described below and delivered to the patient. Typically, the genetic construct which expresses the antisense molecule comprises at least a portion of the said Pax 5 cDNA or gene operatively linked to a promoter which can express the antisense molecule in the cell, preferably urothelial cell, which is or may become cancerous. Promoters that may be active in urothelial cells or cancerous urothelial cells will be known to those skilled in

the art, and may include promoters for ubiquitously expressed, for example housekeeping genes.

Although the genetic construct can be DNA or RNA it is preferred if it is DNA.

Preferably, the genetic construct is adapted for delivery to a human cell.

Means and methods of introducing a genetic construct into a cell in an animal body are known in the art. For example, the constructs of the invention may be introduced into the tumour cells by any convenient method, for example methods involving retroviruses, so that the construct is inserted into the genome of the tumour cell. For example, in Kuriyama *et al* (1991) *Cell Struc. and Func.* **16**, 503-510 purified retroviruses are administered. Retroviruses provide a potential means of selectively infecting cancer cells because they can only integrate into the genome of dividing cells; most normal cells surrounding cancers are in a quiescent, non-receptive stage of cell growth or, at least, are dividing much less rapidly than the tumour cells. Retroviral DNA constructs which encode said antisense agents may be made using methods well known in the art. To produce active retrovirus from such a construct it is usual to use an ecotropic psi2 packaging cell line grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS). Transfection of the cell line is conveniently by calcium phosphate co-precipitation, and stable transformants are selected by addition of G418 to a final concentration of 1 mg/ml (assuming the retroviral construct contains a neo^R gene). Independent colonies are isolated and expanded and the culture supernatant removed, filtered through a 0.45 μm pore-size filter and stored at -70°. For the introduction of the retrovirus into the tumour

cells, it is convenient to inject directly retroviral supernatant to which 10 µg/ml Polybrene has been added. For tumours exceeding 10 mm in diameter it is appropriate to inject between 0.1 ml and 1 ml of retroviral supernatant; preferably 0.5 ml.

Alternatively, as described in Culver *et al* (1992) *Science* 256, 1550-1552, cells which produce retroviruses are injected into the tumour. The retrovirus-producing cells so introduced are engineered to actively produce retroviral vector particles so that continuous productions of the vector occurred within the tumour mass *in situ*. Thus, proliferating tumour cells can be successfully transduced *in vivo* if mixed with retroviral vector-producing cells.

Targeted retroviruses are also available for use in the invention; for example, sequences conferring specific binding affinities may be engineered into preexisting viral *env* genes (see Miller & Vile (1995) *Faseb J.* 9, 190-199 for a review of this and other targeted vectors for gene therapy).

Other methods involve simple delivery of the construct into the cell for expression therein either for a limited time or, following integration into the genome, for a longer time. An example of the latter approach includes (preferably tumour-cell-targeted) liposomes (Nässander *et al* (1992) *Cancer Res.* 52, 646-653).

Immunoliposomes (antibody-directed liposomes) are especially useful in targeting to cancer cell types which over-express a cell surface protein for which antibodies are available. For the preparation of immuno-liposomes MPB-PE (N-[4-(p-maleimidophenyl)butyryl]-phosphatidylethanolamine) is

synthesised according to the method of Martin & Papahadjopoulos (1982) *J. Biol. Chem.* 257, 286-288. MPB-PE is incorporated into the liposomal bilayers to allow a covalent coupling of the antibody, or fragment thereof, to the liposomal surface. The liposome is conveniently loaded with the DNA or other genetic construct of the invention for delivery to the target cells, for example, by forming the said liposomes in a solution of the DNA or other genetic construct, followed by sequential extrusion through polycarbonate membrane filters with 0.6 μm and 0.2 μm pore size under nitrogen pressures up to 0.8 MPa. After extrusion, entrapped DNA construct is separated from free DNA construct by ultracentrifugation at 80 000 x g for 45 min. Freshly prepared MPB-PE-liposomes in deoxygenated buffer are mixed with freshly prepared antibody (or fragment thereof) and the coupling reactions are carried out in a nitrogen atmosphere at 4°C under constant end over end rotation overnight. The immunoliposomes are separated from unconjugated antibodies by ultracentrifugation at 80 000 x g for 45 min. Immunoliposomes may be injected intraperitoneally or directly into the tumour.

Other methods of delivery include adenoviruses carrying external DNA via an antibody-polylysine bridge (see Curiel *Prog. Med. Virol.* 40, 1-18) and transferrin-polycation conjugates as carriers (Wagner *et al* (1990) *Proc. Natl. Acad. Sci. USA* 87, 3410-3414). In the first of these methods a polycation-antibody complex is formed with the DNA construct or other genetic construct of the invention, wherein the antibody is specific for either wild-type adenovirus or a variant adenovirus in which a new epitope has been introduced which binds the antibody. The polycation moiety binds the DNA *via* electrostatic interactions with the phosphate backbone. The adenovirus, because it contains unaltered fibre and penton proteins, is

internalised into the cell and carries into the cell with it the DNA construct of the invention. It is preferred if the polycation is polylysine.

The DNA may also be delivered by adenovirus wherein it is present within the adenovirus particle, for example, as described below.

In the second of these methods, a high-efficiency nucleic acid delivery system that uses receptor-mediated endocytosis to carry DNA macromolecules into cells is employed. This is accomplished by conjugating the iron-transport protein transferrin to polycations that bind nucleic acids. Human transferrin, or the chicken homologue conalbumin, or combinations thereof is covalently linked to the small DNA-binding protein protamine or to polylysines of various sizes through a disulfide linkage. These modified transferrin molecules maintain their ability to bind their cognate receptor and to mediate efficient iron transport into the cell. The transferrin-polycation molecules form electrophoretically stable complexes with DNA constructs or other genetic constructs of the invention independent of nucleic acid size (from short oligonucleotides to DNA of 21 kilobase pairs). When complexes of transferrin-polycation and the DNA constructs or other genetic constructs of the invention are supplied to the tumour cells, a high level of expression from the construct in the cells is expected.

High-efficiency receptor-mediated delivery of the DNA constructs or other genetic constructs of the invention using the endosome-disruption activity of defective or chemically inactivated adenovirus particles produced by the methods of Cotten *et al* (1992) *Proc. Natl. Acad. Sci. USA* 89, 6094-6098 may also be used. This approach appears to rely on the fact that adenoviruses are adapted to allow release of their DNA from an endosome

without passage through the lysosome, and in the presence of, for example transferrin linked to the DNA construct or other genetic construct of the invention, the construct is taken up by the cell by the same route as the adenovirus particle.

This approach has the advantages that there is no need to use complex retroviral constructs; there is no permanent modification of the genome as occurs with retroviral infection; and the targeted expression system is coupled with a targeted delivery system, thus reducing toxicity to other cell types.

It may be desirable to locally perfuse a tumour with the suitable delivery vehicle comprising the genetic construct for a period of time; additionally or alternatively the delivery vehicle or genetic construct can be injected directly into accessible tumours.

It will be appreciated that "naked DNA" and DNA complexed with cationic and neutral lipids may also be useful in introducing the DNA of the invention into cells of the patient to be treated. Non-viral approaches to gene therapy are described in Ledley (1995) *Human Gene Therapy* **6**, 1129-1144.

Alternative targeted delivery systems are also known such as the modified adenovirus system described in WO 94/10323 wherein, typically, the DNA is carried within the adenovirus, or adenovirus-like, particle. Michael *et al* (1995) *Gene Therapy* **2**, 660-668 describes modification of adenovirus to add a cell-selective moiety into a fibre protein. Mutant adenoviruses which replicate selectively in p53-deficient human tumour cells, such as those described in Bischoff *et al* (1996) *Science* **274**, 373-

376 are also useful for delivering the genetic construct of the invention to a cell. Thus, it will be appreciated that a further aspect of the invention provides a virus or virus-like particle comprising a genetic construct of the invention. Other suitable viruses or virus-like particles include HSV, AAV, vaccinia and parvovirus.

In a further embodiment the agent which selectively prevents the function of Pax 5 is a ribozyme capable of cleaving targeted Pax 5 RNA or DNA. A gene expressing said ribozyme may be administered in substantially the same and using substantially the same vehicles as for the antisense molecules.

Ribozymes which may be encoded in the genomes of the viruses or virus-like particles herein disclosed are described in Cech and Herschlag "Site-specific cleavage of single stranded DNA" US 5,180,818; Altman *et al* "Cleavage of targeted RNA by RNAse P" US 5,168,053, Cantin *et al* "Ribozyme cleavage of HIV-1 RNA" US 5,149,796; Cech *et al* "RNA ribozyme restriction endoribonucleases and methods", US 5,116,742; Been *et al* "RNA ribozyme polymerases, dephosphorylases, restriction endonucleases and methods", US 5,093,246; and Been *et al* "RNA ribozyme polymerases, dephosphorylases, restriction endoribonucleases and methods; cleaves single-stranded RNA at specific site by transesterification", US 4,987,071, all incorporated herein by reference.

It will be appreciated that it may be desirable that the antisense molecule or ribozyme is expressed from a urothelial cell-specific promoter element.

The genetic constructs of the invention can be prepared using methods well known in the art.

A variety of methods have been developed to operably link DNA to vectors via complementary cohesive termini. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted to the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

Synthetic linkers containing one or more restriction sites provide an alternative method of joining the DNA segment to vectors. The DNA segment, generated by endonuclease restriction digestion as well know to those skilled in the art, is treated with bacteriophage T4 DNA polymerase or *E. coli* DNA polymerase I, enzymes that remove protruding, 3′-single-stranded termini with their 3′-5′-exonucleolytic activities, and fill in recessed 3′-ends with their polymerising activities.

The combination of these activities therefore generates blunt-ended DNA segments. The blunt-ended segments are then incubated with a large molar excess of linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase. Thus, the products of the reaction are DNA segments carrying polymeric linker sequences at their ends. These DNA segments are then cleaved with the appropriate restriction enzyme and ligated to an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the DNA segment.

Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including International Biotechnologies Inc, New Haven, CN, USA.

A desirable way to modify the DNA encoding the polypeptide of the invention is to use the polymerase chain reaction as disclosed by Saiki *et al* (1988) *Science* 239, 487-491.

In this method the DNA to be enzymatically amplified is flanked by two specific oligonucleotide primers which themselves become incorporated into the amplified DNA. The said specific primers may contain restriction endonuclease recognition sites which can be used for cloning into expression vectors using methods known in the art.

The present invention also relates to a host cell transformed with a genetic (preferably DNA construct) construct of the present invention. The host cell can be either prokaryotic or eukaryotic. Bacterial cells are preferred prokaryotic host cells and typically are a strain of *E. coli* such as, for example, the *E. coli* strains DH5 available from Bethesda Research Laboratories Inc., Bethesda, MD, USA, and RR1 available from the American Type Culture Collection (ATCC) of Rockville, MD, USA (No ATCC 31343). Preferred eukaryotic host cells include yeast and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic cell line. Yeast host cells include YPH499, YPH500 and YPH501 which are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Preferred mammalian host cells include Chinese hamster ovary (CHO) cells available from the ATCC as CCL61, NIH Swiss mouse embryo cells NIH/3T3 available from the ATCC as CRL 1658, and monkey kidney-derived COS-1 cells available from the ATCC as CRL 1650.

Transformation of appropriate cell hosts with a DNA construct of the present invention is accomplished by well known methods that typically depend on the type of vector used. With regard to transformation of prokaryotic host cells, see, for example, Cohen *et al* (1972) *Proc. Natl. Acad. Sci. USA* 69, 2110 and Sambrook *et al* (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. Transformation of yeast cells is described in Sherman *et al* (1986) *Methods In Yeast Genetics, A Laboratory Manual*, Cold Spring Harbor, NY. The method of Beggs (1978) *Nature* 275, 104-109 is also useful. With regard to vertebrate cells, reagents useful in transfecting such cells, for example calcium phosphate and DEAE-dextran or liposome formulations, are available from Stratagene Cloning Systems, or Life Technologies Inc., Gaithersburg, MD 20877, USA.

Electroporation is also useful for transforming cells and is well known in the art for transforming yeast cell, bacterial cells and vertebrate cells.

For example, many bacterial species may be transformed by the methods described in Luchansky *et al* (1988) *Mol. Microbiol.* 2, 637-646 incorporated herein by reference. The greatest number of transformants is consistently recovered following electroporation of the DNA-cell mixture suspended in 2.5X PEB using 6250V per cm at 25μFD.

Methods for transformation of yeast by electroporation are disclosed in Becker & Guarente (1990) *Methods Enzymol.* **194**, 182.

Successfully transformed cells, ie cells that contain a DNA construct of the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of an expression construct

of the present invention can be grown to produce the cytotoxic gene product as defined in the invention. Cells can be harvested and lysed and their DNA content examined for the presence of the DNA using a method such as that described by Southern (1975) *J. Mol. Biol.* 98, 503 or Berent *et al* (1985) *Biotech.* 3, 208. Alternatively, the presence of the protein in the supernatant can be detected using antibodies as described below.

In addition to directly assaying for the presence of recombinant DNA, successful transformation can be confirmed by well known immunological methods when the recombinant DNA is capable of directing the expression of the protein. For example, cells successfully transformed with an expression vector produce proteins displaying appropriate antigenicity. Samples of cells suspected of being transformed are harvested and assayed for the protein using suitable antibodies.

Thus, in addition to the transformed host cells themselves, the present invention also contemplates a culture of those cells, preferably a monoclonal (clonally homogeneous) culture, or a culture derived from a monoclonal culture, in a nutrient medium.

When the genetic construct is a plasmid DNA construct it can be purified. The DNA construct of the invention is purified from the host cell using well known methods.

For example, plasmid vector DNA can be prepared on a large scale from cleaved lysates by banding in a CsCl gradient according to the methods of Clewell & Helinski (1970) *Biochemistry* 9, 4428-4440 and Clewell (1972) *J. Bacteriol.* **110**, 667-676. Plasmid DNA extracted in this way can be

freed from CsCl by dialyse against sterile, pyrogen-free buffer through Visking tubing or by size-exclusion chromatography.

Alternatively, plasmid DNA may be purified from cleared lysates using ion-exchange chromatography, for example those supplied by Qiagen. Hydroxyapatite column chromatography may also be used.

A further aspect of the invention is a method of identifying a suppressor gene or an activator gene for Pax 5 wherein a nucleic acid corresponding to known deletions or duplications in bladder cancer or the product of transcription and/or translation of said nucleic acid is tested to determine whether it may selectively prevent or enhance the function of Pax 5. Thus, the nucleic acid or product may be tested to determine whether it alters the transcription or translation of Pax 5, or affects the stability of Pax 5, or to determine whether it prevents or enhances the binding of Pax 5 to its target DNA. Preferably, the effect on transcription, translation or stability is tested.

A further aspect of the invention provides use of an agent which selectively prevents the function of Pax 5 in the manufacture of a medicament for treating bladder cancer.

A still further aspect of the invention provides a genetic construct comprising a nucleic acid encoding a molecule capable of preventing the function of Pax 5 expressed in a urothelial cell.

As noted above, the genetic construct may be RNA or DNA. The molecule capable of preventing the function of Pax 5 is conveniently an antisense molecule or a ribozyme as disclosed above.

The genetic constructs are adapted for delivery to a human cell, in particular a cell which is cancerous or in which cancer may occur, and more particularly the genetic construct is adapted for delivery to a urothelial cell. The genetic constructs of this aspect of the invention include the viral and non-viral delivery systems described above.

Suitably, the molecule is capable of preventing the function of Pax 5, such as a ribozyme or antisense molecule, is selectively expressed in a bladder cancer cell. For example, expression of said molecule by the genetic construct may be *via* a bladder cancer cell- or tissue-selective promoter which may be a urothelial cell-selective promoter.

A further aspect of the invention provides the genetic constructs for use in medicine. Thus, the genetic constructs are packaged and presented for use in medicine.

A further aspect of the invention provides a pharmaceutical composition comprising a genetic construct of the invention and a pharmaceutically acceptable carrier. The carriers must be "acceptable" in the sense of being compatible with the genetic construct of the invention and not deleterious to the recipients thereof. Typically, the carriers will be water or saline which will be sterile and pyrogen free.

For the avoidance of doubt, the genetic constructs of the invention specifically include virus or virus-like particles.

The invention will now be described by reference to the following Examples and Figures:

Figure 1.

Pax 5 expression in the three established transitional cell carcinoma lines HT-1376, MGH-U1 and RT112.

a. Gel electrophoresis of RTPCR products shows a 500 base pair PCR product in all cell lines examined, visualised by gel electrophoresis and ethidium bromide staining. B-lymphoblasts were used as the positive control.

b. The same RTPCR products visualised by Southern transfer, hybridisation to Pax 5 and autoradiography as described in the Materials and Method section.

Figure 2.

Pax 5 expression in a representative sample of the specimens examined by RT-PCR. Pax 5 expression was detected in 28 out of 35 transitional cell carcinoma samples examined.

a. Gel electrophoresis of RTPCR products for the ubiquitously expressed GAPDH gene expressed in all samples. The 190 base pair GAPDH PCR product is identified. b. Gel electrophoresis of RTPCR, for Pax 5 (500 base pair fragment), shows expression in all of four pT2 samples, four of five pT1, three of five pTa, one of three non-malignant (N58, N56 and N9) and the positive control shown (WEHI-231, a B-lymphoblast cell line).

c. Southern analysis and hybridisation of the same samples confirming that the 500 base pair PCR product is Pax 5.

d. A graphical representation of the trend to a higher proportion of Pax 5 expression in invasive compared to non-invasive transitional carcinoma of the bladder in all of the 29 tumours examined.

e. A histographical representation of the assoication of Pax 5 expression with the grade of the tumour. The number of tumours expressing Pax5 within each grade is displayed as a percentage of the total samples examined in that group. 1/5 nonmalignant samples expressed Pax 5 compared to 4/5 grade 1, 17/21 grade 2 and 8/9 grade 3. Light columns – percentage of tumours expressing Pax 5. Dark columns – percentage of tumours not expressing Pax 5.

Figure 3.

A graphical representation of the relationship between Pax 5 expression and the grade of differentiation of the tumours. There is a clear trend toward Pax 5 expression with increasing dedifferentiation, independent of stage.

Figure 4 (from Adams *et al* (1992))

Sequence and structural organisation of human BSAP (Pax 5) cDNA. A) Entire nucleotide and deduced amino acid sequence of the cDNA clone hBSAP-1. The two peptide sequences obtained by the microsequencing of purified BSAP are highlighted by black overlay. The paired domain is boxed, and the octapeptide (amino acids 179-186) and homeo box homology region (amino acids 229-251) are underlined. The cDNA sequence has GenBank accession No M96944. B) Schematic diagram of the structural organisation of the BSAP-1 cDNA. 5' and 3' untranslated sequences (5'UT and 3'UT, respectively) are indicated by a line, and the long ORF with its subdomains and corresponding amino acid positions is shown by a boxed region.

Figure 5 (from Adams *et al* (1992))

Amino acid sequence comparison of mBSAP (Pax 5), mPax 2 (Dressler *et al* (1990) *Development* **109**, 787-795) and mPax 8 (Plachov *et al* (1990) *Development* **110**, 643-651).

Figure 6.

Pax 5 protein is detected using FITC immunofluorescence in the TCC cell lines MGH-U1, RT112 and HT1376 and the positive control WEHI-231 but not in fibroblasts. This expression pattern reflects that detected using RT-PCR. Santa Cruz antibody sc1974 was used.

A1, A2: fibroblasts. No primary antibody is present in the left hand image. Primary antibody is present in the right hand image. The cells are seen under combined DAPI and FITC filters There is no nuclear signal in either image, confirming that Pax 5 is not expressed in this cell type.

B1, B2: WEHI-231 cells. No primary antibody is present in the left hand image. Primary antibody is present in the right hand image. The cells are seen under combined DAPI and FITC filters Pax 5 expression is shown by the FITC signal localised to the nucleus in image B2.

C1, C2: MGH-U1 cells. In C1, the cells are visualised under the DAPI filter alone. When the filter is switched to FITC, a signal localised to the nucleus is seen. No signal is seen if the primary antibody is omitted (data not shown).

Both RT112 and HT1376 gave a similar appearance to MGH-U1 cells with an FITC signal localised to the nucleus when exposed to the primary antibody (data not shown).

Figure 7.

Pax 2 protein is detected using immunofluorescence in the TCC cell line MGH-U1 and the positive control UOK-132 but not in HT1376. This expression pattern reflects that detected using RT-PCR (Figure 10) in

which expression was found in cell line MHG-U1 but not in RT112 or HT 1376 cells.

A) MGH-U1 cells. In the left hand image, the cells are visualised under the DAPI filter and in the right hand image, the cells are visualised under the FITC filter. The nuclear localisation of the Pax 2 signal confirms the protein expression. No FITC signal was seen under the FITC filter if the primary antibody was replaced by PBS (not shown).

B) The same is demonstrated for UOK-132 cells, again with nuclear localisation of the Pax 2 signal.

C) The cell line HT1376 does not express Pax2 protein, as expected. This also suggests that the positive signals detected in A and B are not due to non-specific binding of the immunoreagents.

Figure 8.

Pax 2 is expressed in 8/35 transitional cell carcinomas and none of the normal urothelium as seen by the 300 bp fragment (using RT-PCR and Southern blot confirmation).

Figure 9.

Pax 8 is expressed in 3 of the 29 tumour samples examined using RT-PCR and Southern hybridisation.

Figure 10.

Pax 2 is expressed in cell line MGH-U1. The arrow marks the position of the 300 base-pair marker which is the expected size of the Pax2 RT-PCR product. MGH-U1 alone appears to express Pax 2 using RT-PCR.

Example 1.

Normal development requires transcription of specific genes in an ordered temporal and spatial manner. This regulated cell growth is in sharp contrast to the deregulated cell growth involved in oncogenesis. This relationship has led to the discovery of a novel mechanism of oncogenesis. Inappropriate or deregulated expression of developmental genes has been shown to play a role in several tumours (7).

The family of nine Pax genes encode nuclear transcription factors and are highly influential in controlling embryonic development. They all contain a "paired box" region of 384 base pairs encoding a DNA binding domain which is highly conserved throughout evolution (8). The influence of Pax genes on developmental processes has been demonstrated by the numerous natural mouse and human syndromes that can be attributed directly to even a heterozygous insufficiency in a Pax gene (9).

The sub-group of Pax 2,5 and 8 (Class III) are expressed latest in development and only in undifferentiated, highly mitotic cells of the ventricular zone of the CNS (Pax 2, 5 and 8), the developing kidney (Pax 2 & 8), B-cell progenitors (Pax 5) and the thyroid (Pax 8). In mice, after birth, Pax 5 is expressed exclusively in B cells and testis (9). These transcription factors are prime candidates for proto-oncogenes because of their powerful effect on cellular growth and differentiation and the salient feature of expression of Pax 2,5 and 8 in highly mitotic, undifferentiated cells. Pax genes are capable of transforming fibroblasts and producing solid, vascular tumours in nude mice (1). They have also been shown to be expressed inappropriately in a number of different human tumours eg glioblastoma (Pax 5), renal cell carcinoma (Pax 2), medulloblastoma, non-Hodgkins lymphoma (Pax 5), Wilms tumour (Pax 2 and 8) and rhabdomyosarcoma (Pax 3 and 7) (reviewed in ref 7).

It has long been thought that deletions on chromosome 9 are an initiating event in urothelial transformation. Comparisons of loss of heterozygosity in tumour and lymphocyte DNA from the same patient has shown that 67% of all bladder tumours, in all stage and grades, have deletions on chromosome 9 (10). The putative tumour suppresser gene lost in these deletions is located at 9p21 and this region is commonly deleted in a variety of tumours (11).

The Pax 5 locus (9p13) lies close to this region and rearrangements at this site may account for inappropriate expression of Pax 5 transcript, producing a growth advantage. Pax 2 has an important role in the pathogenesis of Wilms tumour and renal cell carcinoma and is transcriptionally repressed by the Wilms tumour supressor gene (12). It is therefore quite feasible that the loss of a tumour supressor gene as yet unidentified in bladder cancer could account for the deregulated expression of Pax 5.

Our hypothesis is that Pax 5 expression has a role in the pathogenesis of transitional cell carcinoma of the bladder. We have investigated three established transitional carcinoma cell lines and twenty-nine primary transitional cell carcinomas using reverse transcription polymerase chain reaction to detect Pax 5 expression. We report on the expression of Pax 5 transcripts in transitional cell carcinoma of the human. In our samples, Pax 5 is expressed in a higher proportion of tumours the higher the stage and grade of the disease.

Materials and methods

Cell lines and primary tumour. Three established cell lines MJH-U1 or MGH-U1, HT-1376 and RT112 (kindly donated by Dr John Masters at The Institute of Urology, University College London) were cultured in DMEM with 10% FCS and antibiotics. Twenty-nine transitional cell carcinomas, followed by a further six transitional cell carcinomas, were obtained at transurethral resection performed at Northwick Park and St Marks NHS trust. Half of each tumour biopsy was classified by the histopathologist in the usual clinical setting and half immediately frozen and stored at -70 °C until RNA extraction. Of the original twenty-nine patients, twenty-three were male (79%) and six were female (21%). The mean patient age was 78.3 years. For the group of thirty-five patients considered as a whole, the mean patient age was 77.4 years and the male:female ratio was 4:1.

Non-malignant urothelium. Extracted urothelial RNA from 5 patients with no clinical evidence of neoplasia was kindly donated by Mr Adam Jones from the ICRF at the John Radcliffe hospital, Oxford. The specimens were all obtained at organ retrieval from patients dying in intensive care.

PCR Amplification of Pax 5 cDNA. Total RNA was prepared using TRIZOL® reagent (Life Technologies ™), according to the manufacturers' instructions and in each case 5 µg of total RNA was reverse transcribed using a first-strand cDNA synthesis kit (Pharmacia - protocol according to manufacturers' instructions). PCR amplification for Pax 5 cDNA was performed on the RT product. The ubiquitiously expressed GAPDH was amplified as a control. For amplification of Pax 5 cDNA, the protocol used consisted of 35 cycles of denaturation at 95 °C, annealing at 65 °C and extension at 72 °C. For GAPDH, the annealing temperature was

reduced to 55 °C. PCR primers were designed from the published sequence of Pax 5 (13). Primers used and the size of PCR products were as follows; Pax 5 (500 base pairs), upstream: 5'AGGATGCCGCTGATGGAGTAC 3' and downstream: 5' TGGAGGAGTGAATCAGCTTGG 3'. GAPDH (190 base pairs), upstream: 5'GGCCGTATTGGCGCCTGGTC 3' and downstream 5' GAAGGGCAACTACTGTTCGAAG 3' or upstream 5'ACTGGCGTCTTCACCACCATG3' with downstream 5'GTCATGGATGACCTTGGCCAG3' (194 base pairs). Negative controls were included with water replacing cDNA. The B-lymphoblast cell line WEHI-231 was used as a positive control for Pax 5 expression. The renal cell carcinoma cell line UOK132 was used as a positive control for Pax2 expression.

All the primers were designed so that each annealed within an exon, but the amplified fragment crossed boundaries between exons, so that inadvertent amplification of genomic DNA would include an intron. This would easily be identified by the larger size of the fragment on the gel. Positive controls used were B-lymphoblastoid cells known to express Pax 5, PCR products were electrophoresed on 1.8% agarose gels and transferred to nylon membranes (Qiabrane Nylon Plus, Qiagen). Filters were hybridised with a 1.2 kb human Pax 5 cDNA probe (101691, kindly donated by The Max Planck Institut, Goettingen) labeled with [^{32}P]dCTP using a Rediprime® random primer labelling kit (Amersham) and ExpressHybe® (Clontech) according to the manufacturers' instructions. The filters were then exposed to x-ray film.

Results

Cell lines. All 3 of the established transitional carcinoma cell lines MGH-U1, HT-1376 and RT112 expressed Pax 5 transcript, reproducible on two separate RNA extractions (Fig. 1). MGH-U1 also expressed Pax 2 transcript and protein (Figure 10 and Figure 7), unlike the other two TCC cell lines. None of the TCC cell lines expressed Pax 8 transcript. All three cell lines expressed Pax 5 protein, as shown in relation to MGH-U1 in Figure 6.

Histopathological stage/grade. All 29 tumour specimens were assessed by standard TNM and grade classifications and reviewed by one consultant histopathologist. 12 tumours were classified stage pTa, eight stage pT1 and nine stage pT2. Within each stage classification, tumours were subclassified according to grade. Four tumours were classified grade 1, sixteen grade 2 and nine grade 3 (Table 1).

Pax 5 expression in primary transitional cell carcinoma. Pax 5 cDNA was amplified using PCR and expression was found in 23 of the initial 29 tumours examined (79.3%) or 28 of the 35 (including the six additional tumours; 80%). Pax 5 was expressed in a significantly higher proportion of malignant that benign urothelium ($P=0.02$, Fisher's exact test for the smaller group and $P=0.015$ for the larger group). For the 29 tumours, Pax 5 expression was found in 50% of pTa tumours (mucosa confined) compared to 87.5% of pT1(invading lamina propria) and 89% of pT2 (invading muscle). Pax 5 expression was found in a higher proportion of tumours with increasing stage and this trend reached statistical significance (χ^2 test for trend = 6.885, 1 d.f $p=0.009$) (Fig. 2). Pax 5 expression was also seen in a higher proportion of tumours with increasing de-differentiation, independent of stage. In grade 1, well differentiated tumours 50% expressed Pax 5 compared to 88% of moderately to poorly

differentiated tumours (grades 2/3) and again this trend reached statistical significance (χ^2 test for trend = 8.463 and p=0.004) (Fig.3). Expression of Pax 5 was more frequent in muscle-invasive than in superficial tumours.

Of the five non-malignant urothelial samples studied, Pax 5 transcript was found in only one sample.

Despite the small numbers in this study, there was a significant difference in distribution of Pax 5 expression between non-malignant and malignant urothelium (χ^2 =4.651, p=0.03 with Yates correction 1 d.f.).

Considering the 29 TCC samples and non-malignant samples, the odds ratio for Pax gene expression in malignancy is 15.333; Pax 5 confers a relative risk of 3.97. When the larger group of 35 TCC samples is considered, the relative risk is calculated as 3.71.

Discussion

Several area of molecular research have contributed to the understanding of the initiation and progression of transitional cell carcinoma. Approximately 50% of bladder tumours exhibit loss of p53 function and this is strongly associated with higher tumour stage and grade (14).

Similarly, retinoblastoma gene mutations are seen in about 30% of bladder cancer with propensity to more advanced disease (15, 16). DNA ploidy has also offered some predictive value in superficial disease (17). Many markers of initiation and progression have been investigated, but as yet none can predict outcome as well as the pathologist.

We have studied the expression of Pax 5 in transitional cell carcinoma of the bladder and have demonstrated expression in approximately 80% of the primary tumours studied and all three cell lines.

The Pax 5 protein was first identified by its role in B cell development and was thus called BSAP or B cell specific activator protein (18). Busslinger and colleagues have studied B cell development extensively and unravelled several regulators of Pax 5 expression as well as downstream targets (9). Mice lacking the Pax 5 gene exhibit complete arrest of B cell development at an early stage such that their serum lacks immunoglobulin and the posterior midbrain and anterior cerebellum fail to develop fully (19).

Pax 5 has been shown to be important in the pathogenesis of malignant astrocytoma and is most highly overexpressed in the more aggressive form of the disease, glioblastoma multiforme, suggesting a possible role in progression (20). In this study, Pax 5 expression coincided with the expression of the epidermal growth factor receptor (located on chromosome 7q) in every case. However, treatment of astrocyte cell cultures with EGF did not increase Pax 5 expression, nor did the Pax 5 protein bind the EGFR promoter.

Trisomy 7 is well documented in bladder cancer and is thought to be an early event (21) and EGFR has been shown to be overexpressed in transitional cell carcinoma, especially in higher stage disease (22). An important inverse relationship between Pax 5-expressing astrocytomas and p53 expression led to the discovery that Pax 2, 5 and 8 proteins are capable of binding to a sequence within the 5' regulatory region of the human p53 gene and repressing its activity (23). This novel, proposed

mechanism for down-regulation of the p53 tumour supressor gene without the need for mutation is a possible mechanism for the action of Pax 5 in bladder cancer.

In our study, deregulated expression of Pax 5 in transitional cell carcinoma was found in a higher proportion of poorly differentiated tumours and this is in keeping with expression of Pax 5 in the childhood cerebellar tumour, medulloblastoma. Kozmik and colleagues demonstrated an inverse relationship between expression of Pax 5 and neuronal differentiation (24).

During development, Pax 5 is expressed in the early stages of B cell development but is downregulated rapidly before terminal differentiation: similarly, Pax 2 and 8 are downregulated after the transition from mesenchmye to epithelium during renal development (25). It is interesting to speculate that expression of Pax 5 seen in bladder tumours and medulloblastoma might represent a "reverse mechanism" to that seen in development ie that it is required in order for a terminally differentiated cell to dedifferentiate and adopt a neoplastic phenotype. Constitutive expression of Pax 2, 5 and 8 would keep a cell in an undifferentiated state, thus contributing to its oncogenic potential. Certainly, it has been shown that constitutive expression of Pax 2 in mice, under the control of a CMV promoter, blocks terminal differentiation and the pathological state resembles precancerous lesions (26).

Although we have not established any functional relevance of the Pax 5 gene in transitional cell carcinoma, growth advantage due to its inappropriate expression has been demonstrated in other tumours. In renal cell carcinoma, it was shown that 73% of tumours studied expressed Pax 2

mRNA and protein and that treatment with antisense oligodeoxynucleotides not only downregulated the protein but produced a considerable growth inhibition in culture (27).

In our study, one of five non-malignant urothelial specimens expressed Pax 5: the reason for this is not clear. No role has been demonstrated for Pax 5 in bladder development in mice or humans to date, but we are unaware of any other study that specifically investigated the expression of Pax genes in human bladder. We suspect that some pathological process requiring deregulated Pax 5 expression has occurred in this non-malignant specimen. One theory is that the cell proliferation of the normal urothelium in response to an indwelling catheter (which often has a macroscopic resemblance to bladder tumour) may be expressing Pax 5 but this and other non-malignant pathologies need further investigation. These non-malignant samples had been obtained at organ retrieval from patient donors who died in an intensive care unit, where patients are usually catheterised for some time before death. Investigation of Pax 5 expression may therefore be useful in the diagnosis or monitoring of non-malignant urothelial damage. It is also possible that the "non-malignant" sample was in fact a "early (undetected) malignant" sample in which early malignant changes had occurred but had not been detected clinically.

It has also been reported that Pax 2 expression (normally downregulated after terminal differentiation in renal epithelium) can be upregulated in response to injury eg after surgical obstruction of the ureters in a rat model (28). There may be a similar response to insult in bladder urothelium.

The presence of Pax 5 expression in the samples studied was associated with a four fold relative risk of malignancy and this has far reaching implications for the use of Pax 5 expression as a diagnostic marker and a potential therapeutic target in transitional cell carcinoma. The ease with which urothelial cells can be obtained *via* urine collection suggests that there may be a non-invasive clinical application for this marker.

As shown by Figures 7 to 10, Pax 2 mRNA and protein expression may be detected in transitional cell carcinoma samples and cell lines. Pax 8 expression may be detected in transitional cell carcinoma samples but was not detected in TCC cell lines. 80% (28/35) of the transitional cell carcinomas tested expressed Pax 5, 22.8%(8/35) expressed Pax 2 and 3/29 expressed Pax 8. Pax 5 expression is significantly associated with malignancy using Fisher's Exact test. Detection of Pax 5 expression may therefore be useful in diagnosis of bladder cancer.

Table 1.

Pax 5 expression in five non-malignant human urothelial specimens examined and in 29 transitional cell carcinomas.

The malignant specimens are further grouped according to histological stage and grade.

Histological stage/grade	No of tumours analysed	Pax 5 +ve	Pax 5 -ve
Non-malignant	5	1	4
PTa(G1/G2/G3)	12(3/9/0)	8(2/6/0)	4(1/3/0)
PT1(G1/G2/G3)	8(0/4/4)	7(0/4/3)	1(0/0/1)
PT2(G1/G2/G3)	9(1/3/5)	8(0/3/5)	1(1/0/0)
G1	4	2	2
G2	16	13	3
G3	9	8	1

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Example 2.

A screen may be carried out using either of the following methods:

An urine sample is obtained from a patient at risk of bladder cancer for occupational reasons. The urine sample is centrifuged in order to sediment the cells, including urothelial cells, shed into the urine. The sedimented cells are retained and cDNA prepared from them as described in Example 1 above. PCR analysis is conducted on the prepared cDNA as described in Example 1 above.

Alternatively, the sedimented cells obtained as described above are deposited on a slide in a similar manner to that used when performing histological analysis of cells shed into urine. The cells are then exposed to an anti-Pax 5 antibody so that the antibody binds to Pax 5 present in the cells and the bound antibody is detected, using techniques well known in cytology laboratory diagnostic testing. The results are presented as the presence of Pax 5 positive cells, or as the proportion of the cells examined that are Pax 5 positive, or in any other useful way.

If Pax 5 expression is detected, histological and other examinations are carried out in order to determine whether the patient has bladder cancer, and if so, of what grade. An appropriate course of treatment may then be adopted. If there is no sign of bladder cancer, the patient may be monitored at regular intervals in order to detect any cancerous changes as early as possible.

CLAIMS

1. A method of determining the susceptibility of a human patient to bladder cancer comprising the steps of (i) obtaining a sample containing nucleic acid and/or protein from urothelial of the patient; and (ii) determining whether the sample contains a level of Pax 5 nucleic acid or protein associated with bladder cancer.

2. A method of diagnosing bladder cancer in a human patient comprising the steps of (i) obtaining a sample containing nucleic acid and/or protein from urothelial cells of the patient; and (ii) determining whether the sample contains a level of Pax 5 nucleic acid or protein associated with bladder cancer.

3. A method of predicting the relative prospects of a particular outcome of bladder cancer in a human patient comprising the steps of (i) obtaining a sample containing nucleic acid and/or protein from the urothelium of the patient; and (ii) determining whether the sample contains a level of Pax 5 nucleic acid or protein associated with bladder cancer.

4. A method according to any one of Claims 1 to 3 wherein the bladder cancer is transitional cell carcinoma of the bladder.

5. A method according to any one of claims 1 to 4 wherein the cancer is invasive.

6. A method according to any one of the preceding claims wherein the sample contains nucleic acid and the level of Pax 5 nucleic acid is

measured by contacting the said nucleic acid with a nucleic acid which hybridises selectively to Pax 5 nucleic acid.

7. A method according to Claim 6 wherein the sample contains mRNA and the nucleic acid as said selectively hybridises to Pax 5 mRNA.

8. A method according to Claim 6 or 7 wherein the nucleic acid which hybridises as said is detectably labelled.

9. A method according to any one of Claims 6 to 8 wherein the nucleic acid which selectively hybridises as said is single stranded.

10. A method according to any one of Claims 6 to 9 wherein the nucleic acid which selectively hybridises as said is suitable for use in a nucleic acid amplification reaction.

11. A method according to any one of Claims 1 to 5 wherein the sample contains protein and the level of Pax 5 protein is measured.

12. A method according to Claim 11 wherein the level of said protein is measured by contacting the protein with a molecule which selectively binds to Pax 5 protein.

13. A method according to Claim 12 wherein the selective binding molecule is an antibody or fragment or derivative thereof or an antibody-like molecule.

14. A method according to Claim 12 or 13 wherein the selective binding molecule comprises a detectable label.

15. A method according to Claim 11 wherein the level of Pax 5 is measured by selectively assaying its activity in the sample.

16. A method according to any one of claims 1 to 15 wherein the sample is a sample of the tissue in which bladder cancer is suspected or in which bladder cancer may be or has been found, or contains cells from said tissue.

17. A method according to Claim 16 wherein the tissue is urothelium and the cancer is transitional cell carcinoma of the bladder.

18. A method according to Claim 16 wherein the sample is urine and the cancer is transitional cell carcinoma of the bladder.

19. Use of an agent which is capable of use in determining the level of Pax 5 protein or nucleic acid in a sample in the manufacture of a reagent for diagnosing bladder cancer.

20. Use according to Claim 19 wherein the agent is a nucleic acid which selectively hybridises to Pax 5 nucleic acid.

21. Use according to Claim 19 wherein the agent is a molecule which selectively binds to Pax 5 protein.

22. Use according to Claim 21 wherein the agent is useful in selectively assaying the activity of Pax 5 protein.

23. Use of an agent as defined in any one of Claim 19 to 22 in a method of diagnosing bladder cancer.

24. Use of an agent as defined in any one of Claim 19 to 22 for diagnosing bladder cancer.

25. A kit of parts useful for diagnosing bladder cancer comprising an agent which is capable of use in determining the level of Pax 5 protein or nucleic acid in a sample and a control sample wherein the control sample may be a negative control not comprising a detectable amount of Pax 5 nucleic acid or protein, or it may be a positive control comprising a detectable amount of Pax 5 nucleic acid or protein.

26. A method of treating bladder cancer comprising the step of administering to the patient an agent which selectively prevents the function of Pax 5.

27. A method according to Claim 26 wherein the agent prevents the expression of Pax 5.

28. A method according to Claim 26 wherein the agent inhibits the activity of Pax 5.

29. A method according to Claim 27 wherein the agent is an antisense molecule.

30. A method according to Claim 28 wherein the agent is a ribozyme.

31. Use of an agent which selectively prevents the function of Pax 5 in the manufacture of a medicament for treating bladder cancer.

32. A genetic construct comprising a nucleic acid encoding a molecule capable of preventing the function of Pax 5 expressed in a urothelial cell.

33. A genetic construct according to Claim 32 adapted for delivery to a human urothelial cell.

34. A genetic construct according to Claim 33 wherein the adaptation allows delivery to a bladder cancer cell.

35. A genetic construct according to Claim 33 or 34 comprising means to selectively deliver the nucleic acid to a bladder cancer cell.

36. A genetic construct according to any one of Claims 32 to 35 comprising means to selectively express the nucleic acid encoding a molecule as said in a bladder cancer cell.

37. A genetic construct according to any one of Claims 32 to 36 for use in medicine.

38. A pharmaceutical composition comprising a genetic construct according to any one of Claims 32 to 36 and a pharmaceutically acceptable carrier.

39. Any novel method of treating or diagnosing bladder cancer as herein disclosed.

40. Any novel composition for use in treating or diagnosing bladder cancer as herein disclosed.

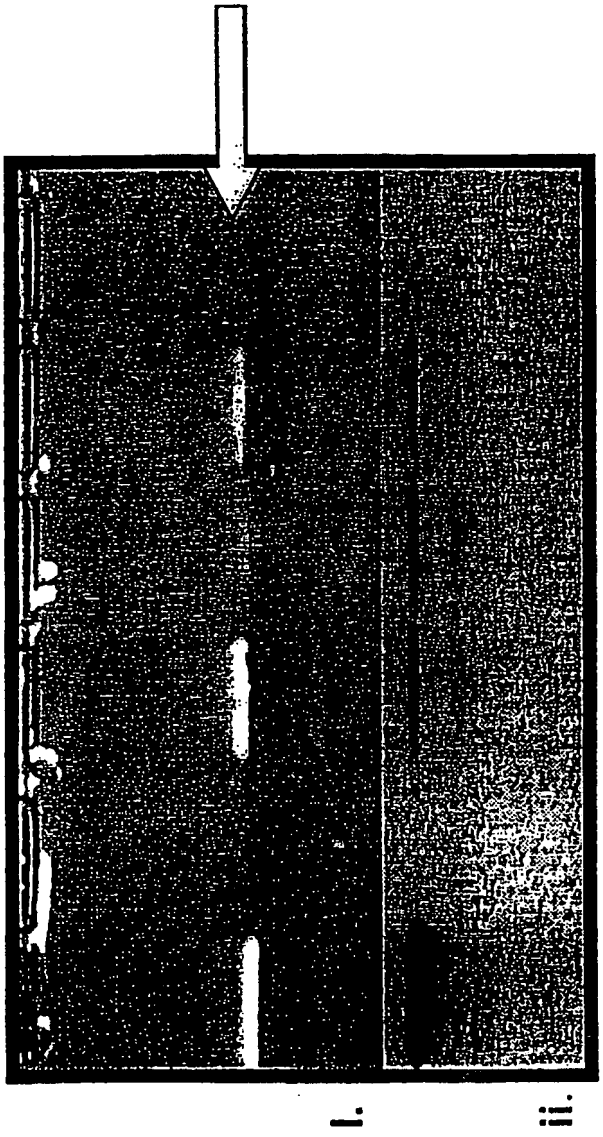

Figure 1

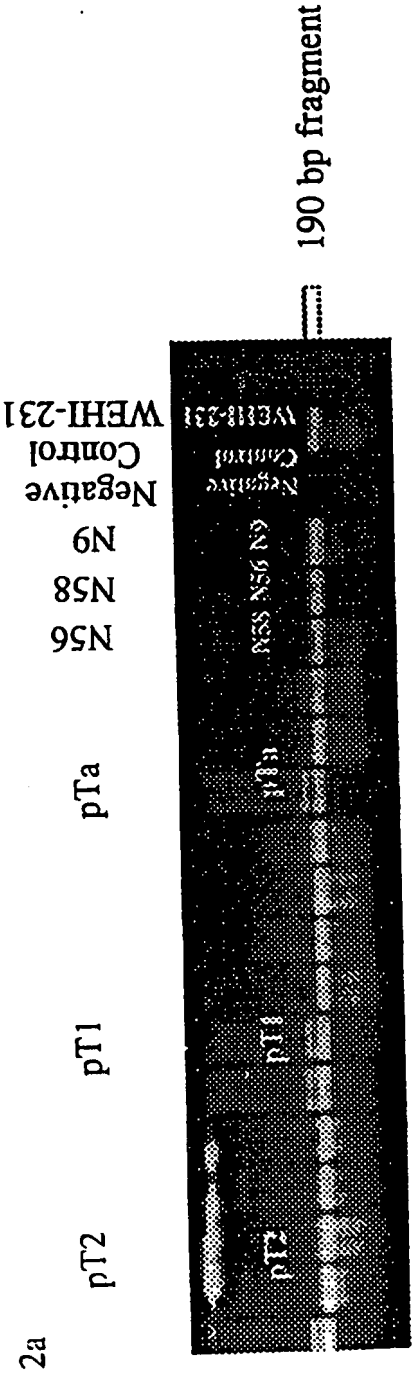

Figure 2a

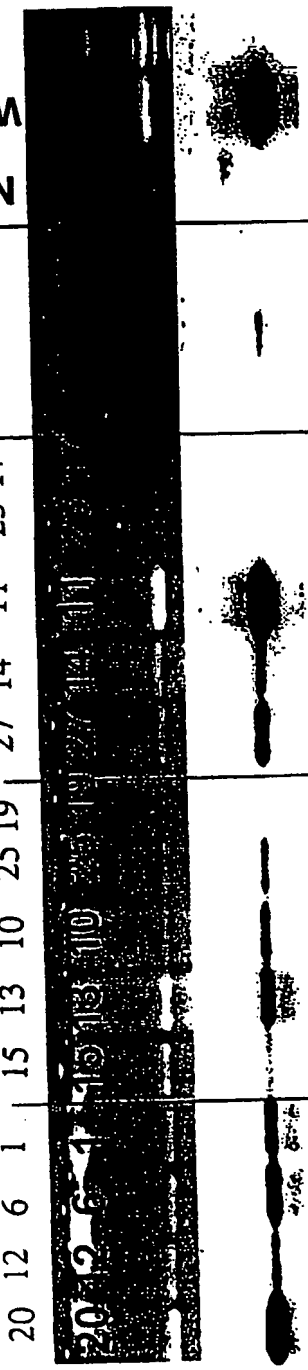

Figure 2b

Figure 2c

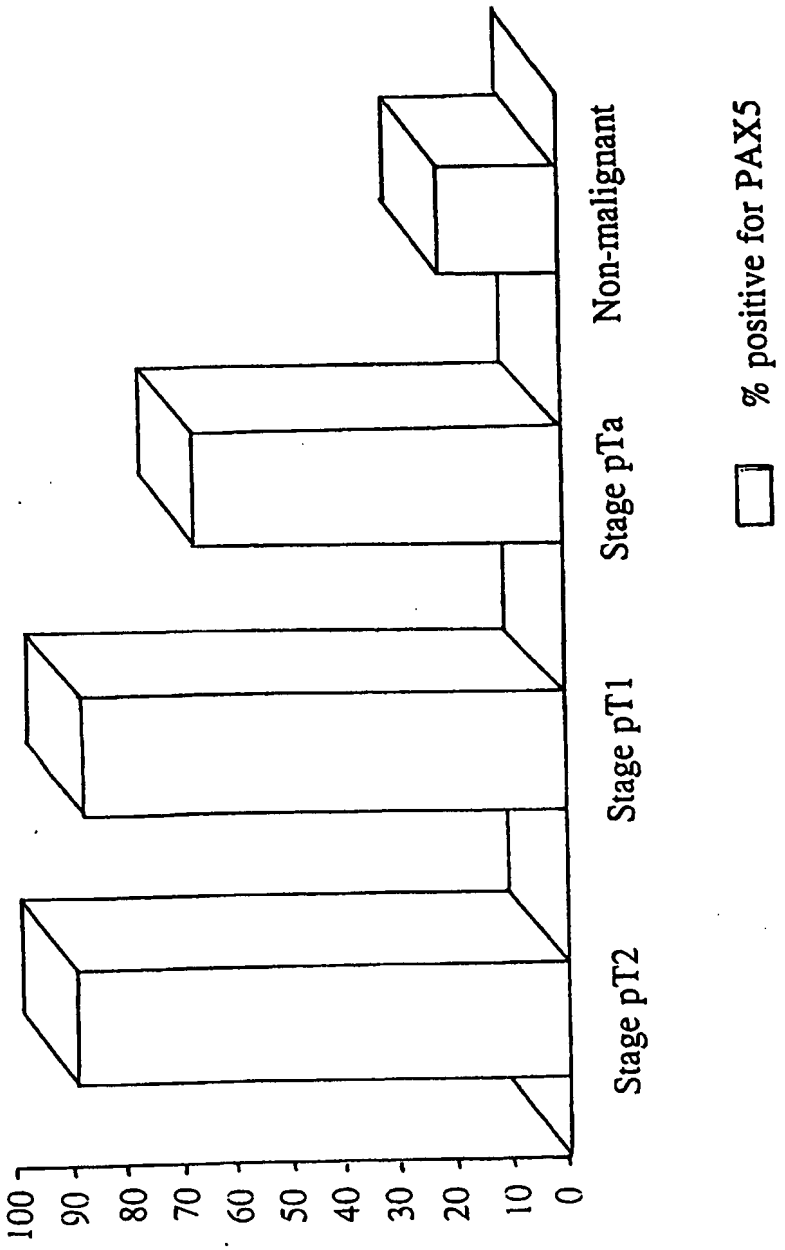
100
90
80
70
60
50
40
30
20
10
0
Stage pT2
Stage pT1
Stage pTa
Non-malignant
% positive for PAX5

Figure 2d

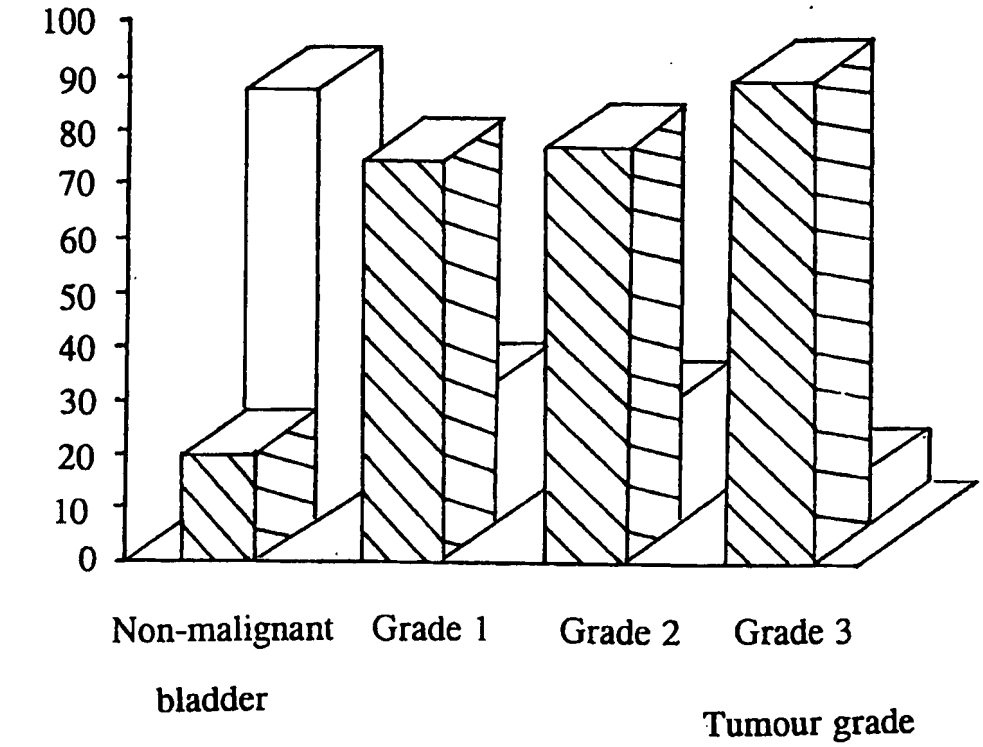
Percentage of tumours
100
90
80
70
60
50
40
30
20
10
0
Non-malignant bladder
Grade 1
Grade 2
Grade 3
Tumour grade
Percentage of tumours expressing PAX5
Percentage of tumours not expressing PAX5

Figure 2e

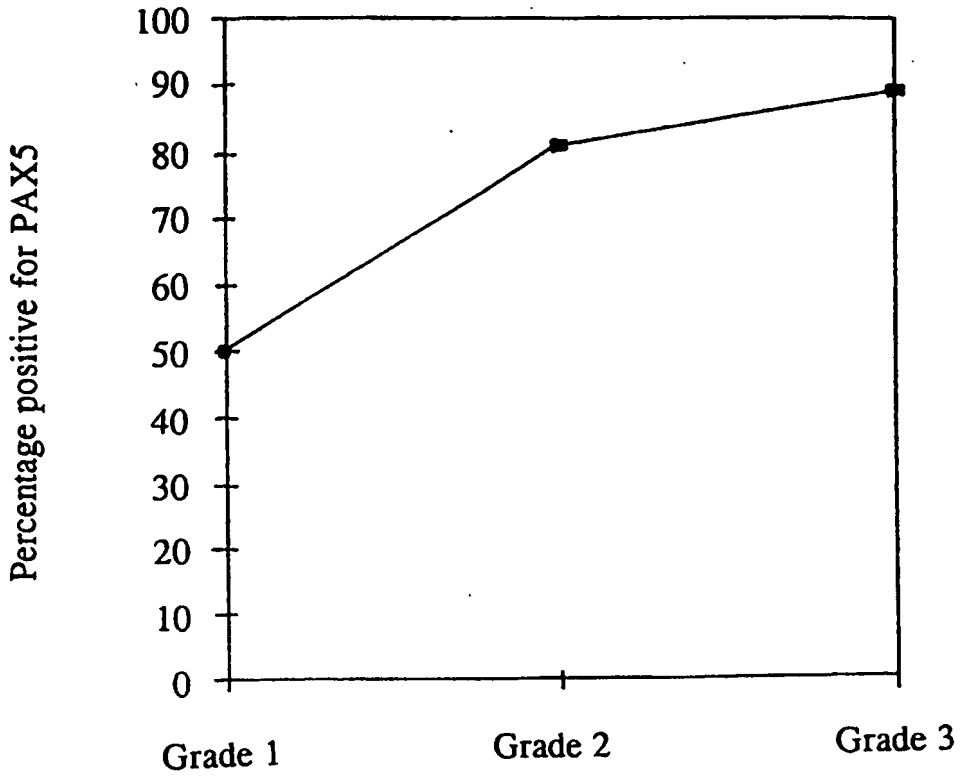
Percentage positive for PAX5
100
90
80
70
60
50
40
30
20
10
0
Grade 1
Grade 2
Grade 3

Figure 3

A

```
1   AAAAAAAAGAAAAAAAAAGGCACAAAAAAGTGGAAACTTTTCCCTGTCCATTCCATCAAGTCCTGAAAAATCAAAATGGATTTAGAGAAA
                                                                    M  D  L  E  K

91  AATTATCCGACTCCTCGGACCAGCAGGACAGGACATGGAGGAGTGAATCAGCTTGGGGGGGTTTTTGTGAATGGACGGCCACTCCCGGAT
6   N  Y  P  T  P  R  T  S  R  T  G  H  G  G  V  N  Q  L  G  G  V  F  V  N  G  R  P  L  P  D

181 GTAGTCCGCCAGAGGATAGTGGAACTTGCTCATCAAGGTGTCAGGCCCTGCGACATCTCCAGGCAGCTTCGGGTCAGCCATGGTTGTGTC
36  V  V  R  Q  R  I  V  E  L  A  H  Q  G  V  R  P  C  D  I  S  R  Q  L  R  V  S  H  G  C  V

271 AGCAAAATTCTTGGCAGGTATTATGAGACAGGAAGCATCAAGCCTGGGGTAATTGGAGGATCCAAACCAAAGGTCGCCACACCCAAAGTG
66  S  K  I  L  G  R  Y  Y  E  T  G  S  I  K  P  G  V  I  G  G  S  K  P  K  V  A  T  P  K  V

361 GTGGAAAAAATCGCTGAATATAAACGCCAAAATCCCACCATGTTTGCCTGGGAGATCAGGGACCGGCTGCTGGCAGAGCGGGTGTGTGAC
96  V  E  K  I  A  E  Y  K  R  Q  N  P  T  M  F  A  W  E  I  R  D  R  L  L  A  E  R  V  C  D

451 AATGACACCGTGCCTAGCGTCAGTTCCATCAACAGGATCATCCGGACAAAAGTACAGCAGCCACCCAACCAACCAGTCCCAGCTTCCAGT
126 N  D  T  V  P  S  V  S  S  I  N  R  I  I  R  T  K  V  Q  Q  P  P  N  Q  P  V  P  A  S  S

541 CACAGCATAGTGTCCACTGGCTCCGTGACGCAGGTGTCCTCGGTGAGCACGGATTCGGCCGGCTCGTCGTACTCCATCAGCGGCATCCTG
156 H  S  I  V  S  T  G  S  V  T  Q  V  S  S  V  S  T  D  S  A  G  S  S  Y  S  I  S  G  I  L

631 GGCATCACGTCCCCCAGCGCCGACACCAACAAGCGCAAGAGAGACGAAGGTATTCAGGAGTCTCCGGTGCCGAACGGCCACTCGCTTCCG
186 G  I  T  S  P  S  A  D  T  N  K  R  K  R  D  E  G  I  Q  E  S  P  V  P  N  G  H  S  L  P

721 GGCAGAGACTTCCTCCGGAAGCAGATGCGGGGAGACTTGTTCACACAGCAGCAGCTGGAGGTGCTGGACCGCGTGTTTGAGAGGCAGCAC
216 G  R  D  F  L  R  K  Q  M  R  G  D  L  F  T  Q  Q  Q  L  E  V  L  D  R  V  F  E  R  Q  H

811 TACTCAGACATCTTCACCACCACAGAGCCCATCAAGCCCGAGCAGACCACAGAGTATTCAGCCATGGCCTCGCTGGCTGGTGGGCTGGAC
246 Y  S  D  I  F  T  T  T  E  P  I  K  P  E  Q  T  T  E  Y  S  A  M  A  S  L  A  G  G  L  D
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Figure 4 (page 1 of 3)

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 901  GACATGAAGGCCAATCTGGCCAGCCCCACCCCTGCTGACATCGGGAGCAGTGTGCCAGGCCCGCAGTCCTACCCCATTGTGACAGGCCGT
 276  D  M  K  A  N  L  A  S  P  T  P  A  D  I  G  S  S  V  P  G  P  Q  S  Y  P  I  V  T  G  R

 991  GACTTGGCGAGCACGACCCTCCCCGGGTACCCTCCACACGTCCCCCCCGCTGGACAGGGCAGCTACTCAGCACCGACGCTGACAGGGATG
 306  D  L  A  S  T  T  L  P  G  Y  P  P  H  V  P  P  A  G  Q  G  S  Y  S  A  P  T  L  T  G  M

1081  GTGCCTGGGAGTGAGTTTTCCGGGAGTCCCTACAGCCACCCTCAGTATTCCTCGTACAACGACTCCTGGAGGTTCCCCAACCCGGGGCTG
 336  V  P  G  S  E  F  S  G  S  P  Y  S  H  P  Q  Y  S  S  Y  N  D  S  W  R  F  P  N  P  G  L

1171  CTTGGCTCCCCCTACTATTATAGCGCTGCCGCCCGAGGAGCCGCCCCACCTGCAGCCGCCACTGCCTATGACCGTCACTGACCCTTGGAG
 366  L  G  S  P  Y  Y  Y  S  A  A  A  R  G  A  A  P  P  A  A  A  T  A  Y  D  R  H  *

1261  CCAGGCGGGCACCAAACACTGATGGCACCTATTGAGGGTGACAGCCACCCAGCCCTCCTGAAGATAGCCAGAGAGCCCATGAGACCGTCC
1351  CCCAGCATCCCCCACTTGCCTGAAGCTCCCCTCTTCCTCTCTTCCTCCAGGGACTCTGGGGCCCTTTGGTGGGGCCGTTGGACTTCTGGA
1441  TGCTTGTCTATTTCTAAAAGCCAATCTATGAGCTTCTCCCGATGGCCACTGGGTCTCTGCAAACCAATAGACTGTCCTGCAAATAACCGC
1531  AGCCCCAGCCCAGCCTGCCTGTCCTCCAGCTGTCTGACTATCCATCCATCATAACCACCCCAGCCTGGGAAGGAGAGCTTGCTTTTGTTG
1621  CTTCAGCAGCACCCATGTAAATACCTTCTTGCTTTTCTGTGGGCCTGAAGGTCCGACTGAGAAGACTGCTCCACCCATGATGCATCTCGC
1711  ACTCTTGGTGCATCACCGGACATCTTAGACCTATGGCAGAGCATCCTCTCTGCCCTGGGTGACCCTGGCAGGTGCGCTCAGAGCTGTCCT
1801  CAAGATGGAGGATGCTGCCCTTGGGCCCCAGCCTCCTGCTCATCCCTCCTTCTTTAGTATCTTTACGAGGAGTCTCACTGGGCTGGTTGT
1891  GCTGCAGGCTCCCCCTGAGGCCCCTCTCCAAGAGGAGCACACTTTGGGGAGATGTCCTGGTTTCCTGCCTCCATTTCTCTGGGACCGATG
1981  CAGTATCAGCAGCTCTTTTCCAGATCAAAGAACTCAAAGAAAACTGTCTGGGAGATTCCTCAGCTACTTTTCCGAAGCAGAATGTCATCC
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2341  ATCACAAACAGCCTACAGCCAGAACTTCTGAGCATCCTCTCGGGGCAGACCCTCCCCGTCCTCGTGGAGCTTAGCAGGCAGCTGGGCATG
2431  GAGGTGCTGGGGCTGGGGCAGATGCCTAATTTCGCACAATGCATGCCCACCTGTTGATCTAAGGGGCCGCGATGGTCAGGGCCACGGCCA
2521  AGGGCCACGGGAACTTGGAGAGGGAGCTTGGAGAACTCACTGTGGGCTAGGGTGGTCAGAGGAAGCCAGCAGGGAAGATCTGGGGGACAG
2611  AGGAAGGCCTCCTGAGGGAGGGGCAGGAGAGCAGTGAGGAGCTGCTGTGTGACCTGGGAGTGATTTTGACATGGGGGTGCCAGGTGCCAT
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Figure 4 (page 2 of 3)

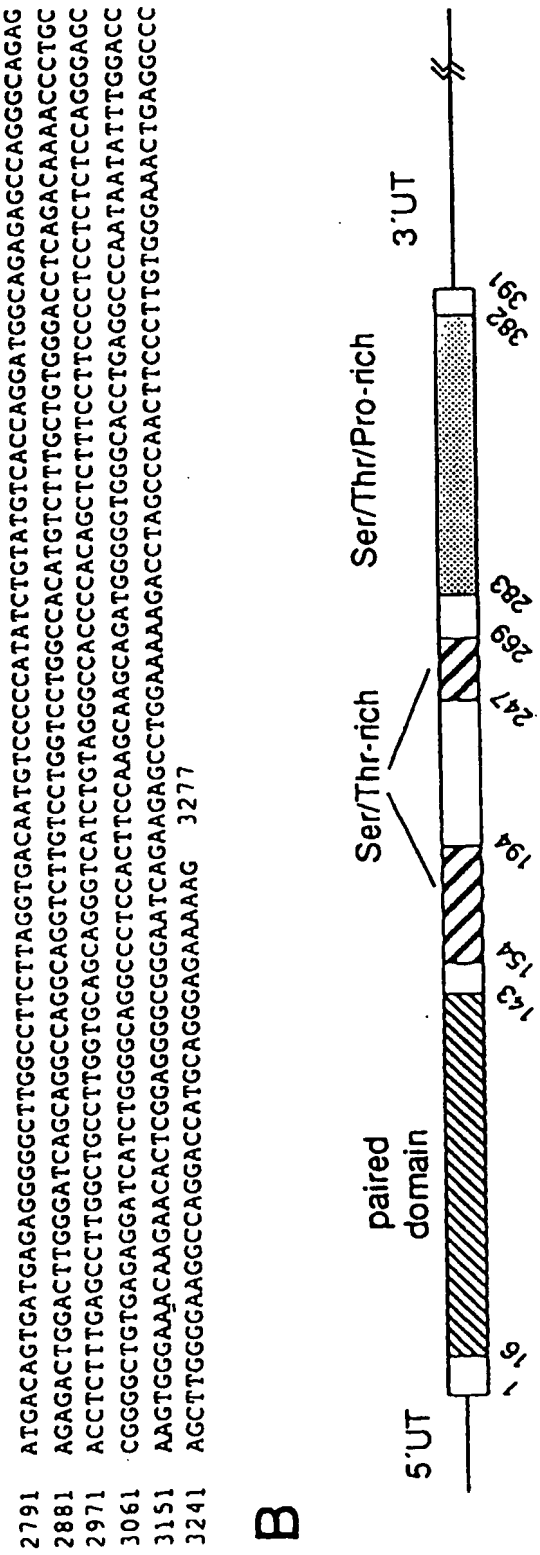

Figure 4 (page 3 of 3)

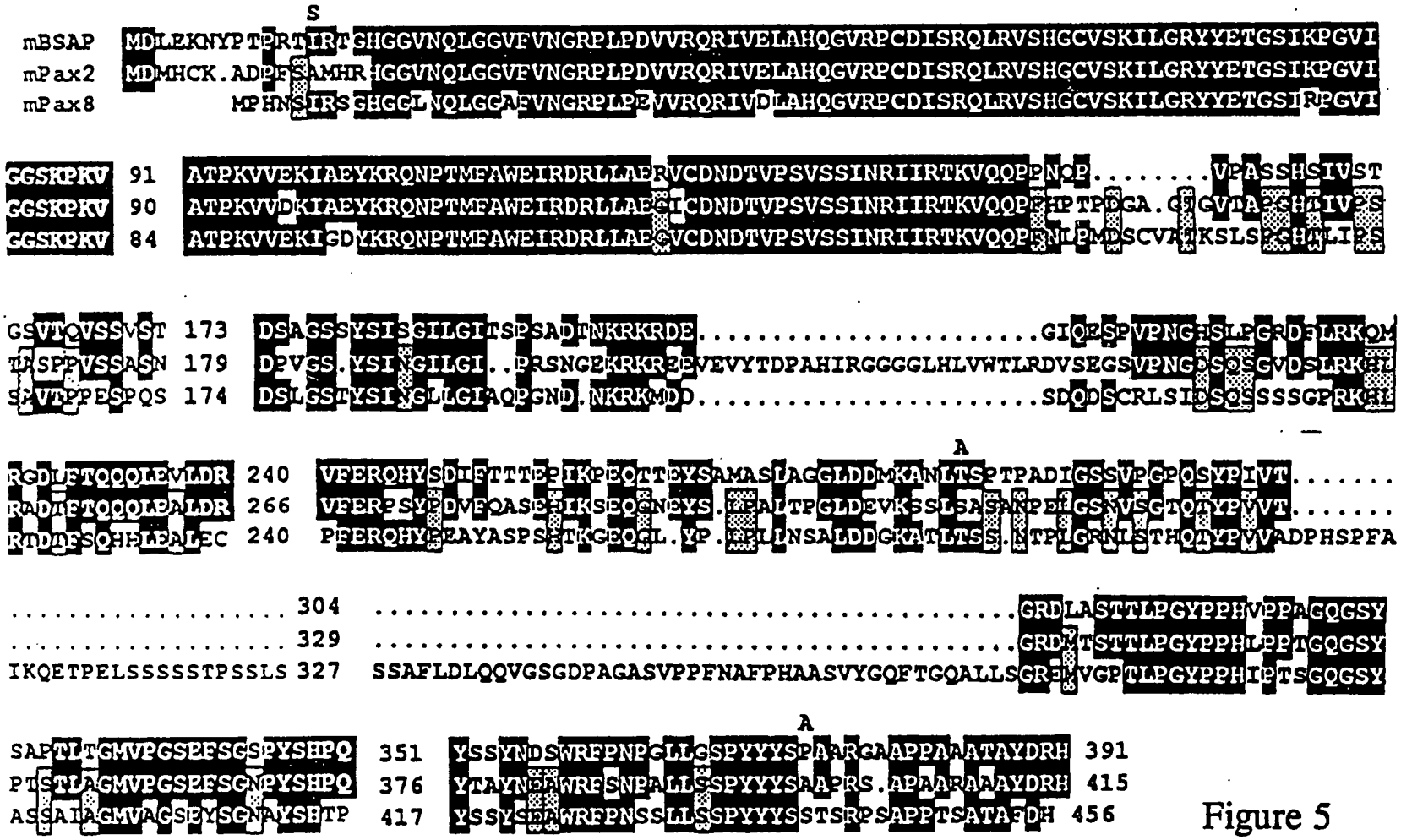

Figure 5

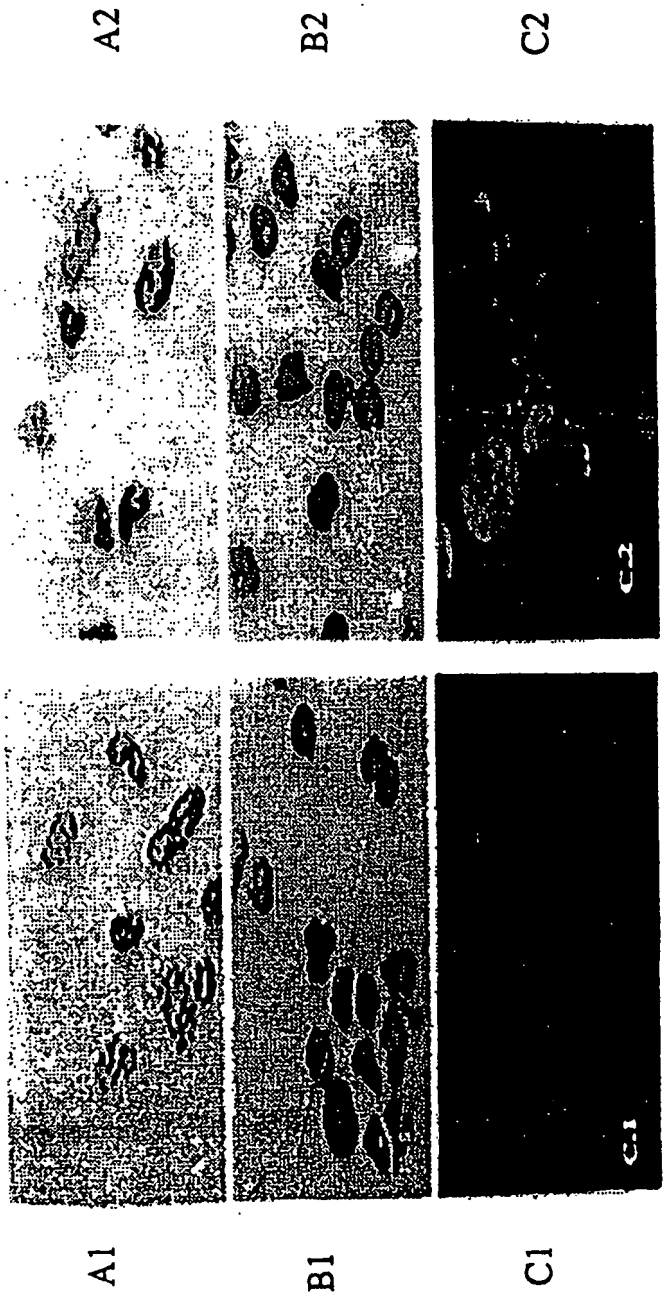

Figure 6

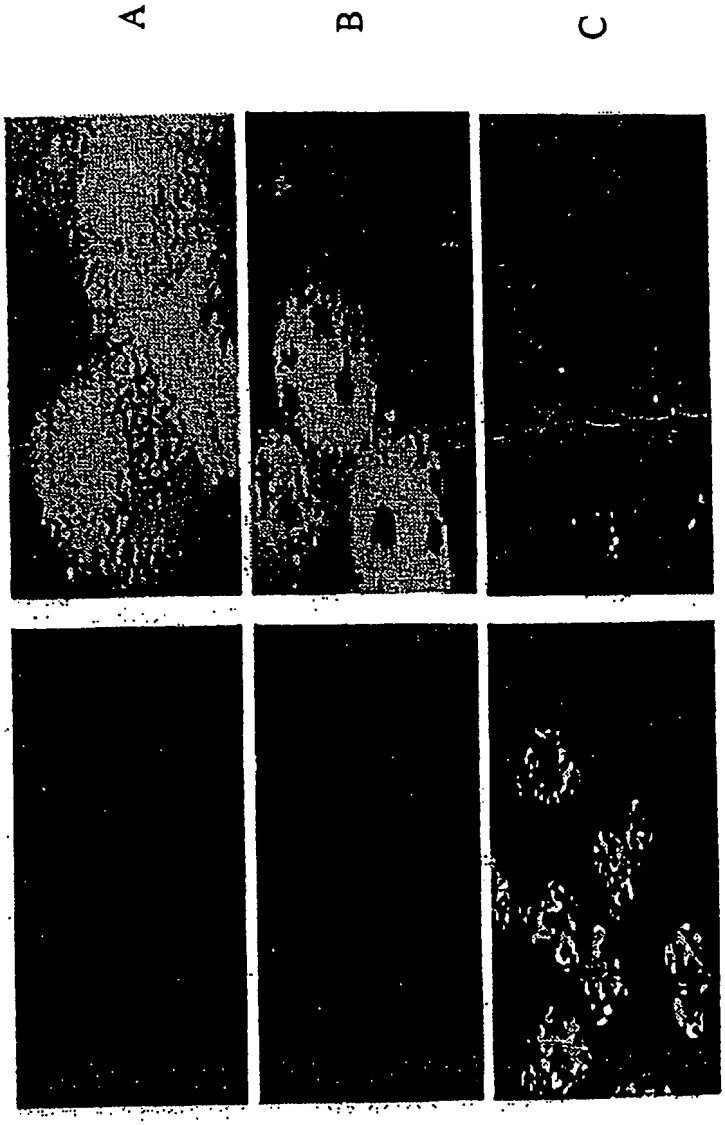

Figure 7

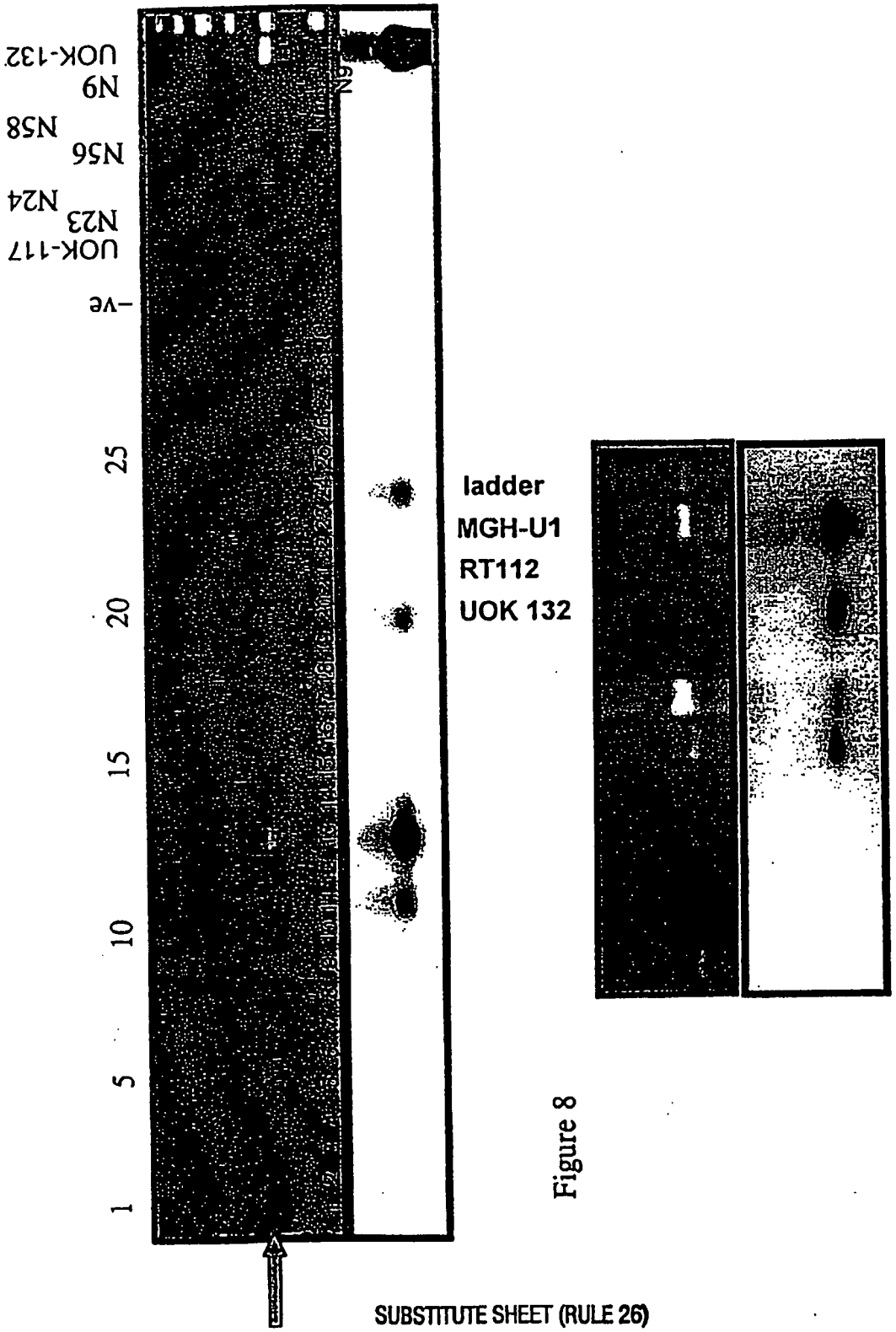
UOK-132
N9
N58
N56
N24
N23
UOK-117
-ve
25
20
15
10
5
1
ladder
MGH-U1
RT112
UOK 132

Figure 8

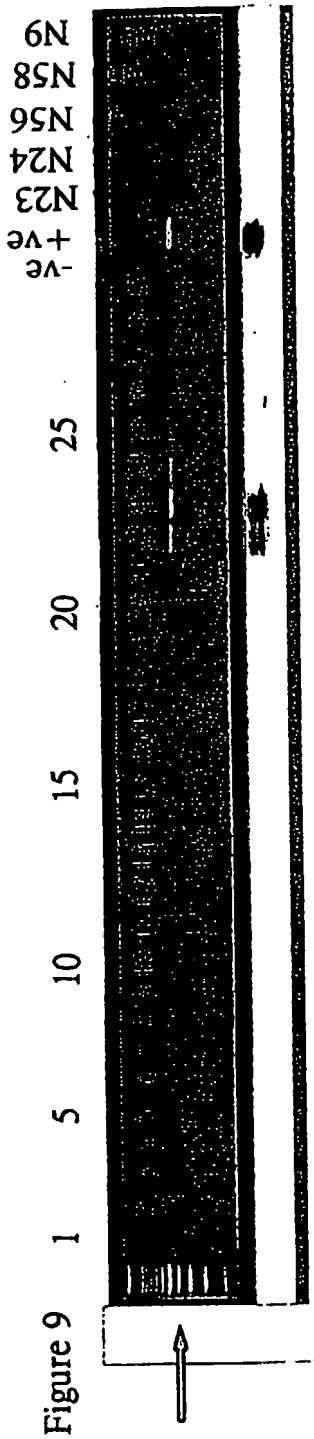

Figure 9

Figure 10